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In re application of

Sergio Rosini

Giorgio Staibano

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For: Pharmacologically active biphosphonates, process for the preparation thereof and pharmaceutical compositions therefrom.

DECLARATION UNDER RULE 132

Sergio Rosini declares:

I graduated in Biological Sciences at the University of Pisa, Italy, in 1968. I work in Istituto Farmaceutico Gentili as Director of the Research Center. I am a member of Società Italiana del Metabolismo Minerale (Italian Society of Mineral Metabolism), of the American Society for Microbiology, of Società Italiana di Farmacologia e tossicologia (Italian Society of Pharmacology and Toxicology). As Director of the Research Center, I am responsible for the pharmacological and toxicological researches, as well as for the researches of clinical pharmacology.

In this capacity I had the opportunity to follow exhaustively all the pharmacological experiments carried out with the compounds that are the subject of the above-identified application.

The diphosphonates belong to a new class of pharmacological agents introduced into the clinical practice during the last years for the treatment of the diseases characterized by a relative or absolute increase of bone reabsorption. Their administration causes a quick dicrease of hydroxyprolinuria and calciuria, parameters related to bone reabsorption, and a slower decrease of alkaline phosphatasemia, which is an index of osteoblastic activity and therefore of bone neoformation.

The up-to-now studied diphosphonates are ethidronate, chlodronate, amino-propane diphosphonate (APD), amino-butane diphosphonate (AHBuBP).
Although all these compounds possess a similar antiosteoclastic activity, they are markedly different as far as the mechanism of action, the dose- effect ratio and the side effects are concerned.

The amino derivatives (amino-propane; amino-butane) are generally much more active than the others, and the pharmacological investigations carried on by Prof. Fleisch (Institut of Pathophysiology of Berne CH) haveshown (Table 1) that AHBuBP has an activity more than 100 times higher than that of Cl_2 M B P (Clodronate). Also in comparison with other amino-derivatives like A P D the AHB uBP has shown an activity 10 times higher and more it should be noted that the clinical use of A P D has been limited by the observation that it causes a decrease in blood lymphocytes and the occurrence of fever.

On these grounds, we have submitted product AHBuBP to clinical investigations on tens of patients affected by tumoral osteolisis and mieloma, and we have been able to ascertain that doses of 1 or 2.5 mg/die allow a positive response to the drug, whereas in order to have similar responses doses of at least 300 mg/die of Clodronate (Cl_2MDP) must be administered.
Besides this difference in the activity of the two drugs, it was also noted that AHBuBP displayed a surprisingly and unforeseeably longer activity than Cl_2 MBP. As a matter of fact, a 5-6 day treatment cause positive effects that last for weeks or months after treatment, whereas Cl_2MBP must be administered continuously in order to maintain the therapeutic effect.
This characteristic has been observed till now only for AHBuBP, the high activity of which could not be foreseen on the grounds of the experiences carried out on diphosphonates in the past.

In particular, we have studied 8 patients affected by multiple mieloma (MM) with diffused osteolitic lesions and strong bone pain. Hypercalcemia, calciuria, creatinemia, phosphatemia, serum alkaline phosphatase and hydroxyprolinuria were accurately monitored in all the patients. The patients were submitted to ABDP treatment, in a dose of 2.5 mg i.v./die for 5 days every third-fourth month, in addition with VCAP polychemotherapy. All the patients noted a remarkable improvement or disappearance of bone pains within the first 5-6 days from the beginning of ABDP administration. Hydroxyprolinuria, calciuria, hypercalcemia - the main signs of bone reabsorption -, that were high or at the upper limits before the treatment, reached normal limits during and after ABDP administration. The progress of osteolitic lesions ceased and sometimes their extension decreased; we have also evidence of the recomposition of pathologic fractures in 2 cases. No side effects were observed. Therefore, we think that ABDP is effective in reducing the extension of osteolisis of MM, and probably also in delaying the formation of new osteolitic foci: moreover, the lowering or block of bone absorption, and therefore the remission of pain seem to persist for a long time after the administration of the drug is discontinued; while other diphosphonates have an activity limited to the period of treatment.

TABLE 1

Amount in mg P/kg	0.01 s.c.	0.1 s.c.	1.0 s.c.	10.0 s.c.	2.0 p.o.
NaCl Controls			29.72 (17) ±3.82		28.45 (12) ±3.22
Cl_2MBP		38.21 (6) ±5.31	47.00 (6) ±4.69	52.65 (6) ±5.64	31.20 (8) ±2.45
AHPrBP	39.53 (7) ±3.60	50.31 (6) ±5.08	------	--------	33.72 (6) ±6.51
AHBuBP	48.96 (7) ±3.82	58.17 (5) ±2.55	60.00 (4) ± 5.78	--------	43.80 ±10.48 (7)
AHPeBP	37.85 (7) ±5.03	50.44 (6) ±5.36	56.47 (6) ±4.08	--------	38.70 ±6.15 (6)
AHHexBP	38.12 (7) ±1.95	46.96 (5) ±5.32	54.93 (5) ±5.91	--------	34.69 ±7.38 (9)

Effect of various aminobisphosphonates administered for 7 days to rats on the metaphyseal density (% volume of calcified tissue).

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

26-8-'85
Date

Signature

Universität Bern
Pathophysiologisches Institut

CH-3010 Bern, Murtenstrasse 35
Telefon 031 64 25 17

Bern, August 24, 1984

Dr. Sergio Rosini
Istituto Gentili S.p.A.
Industria Chimico-Farmaceutica
Via Mazzini 112

I-56100 P I S A

Dear Dr. Rosini,

This letter is to answer your question about the position of 4-amino-1-hydroxybutylidene-1,1-bisphosphonate (AHBuBP) with respect to other bisphosphonates.

As you know from the results I sent you earlier, AHBuBP was found to be extremely active in inhibiting bone resorption in the rat. The effect was tested by measuring morphologically in a quantitative way the amount of bone present in the metaphysis after the administration during 7 days of the compounds. In this test system, AHBuBP was active already at a concentration of 0.001 mg P/kg given s.c. AHBuBP was about ten times more active than the other bisphosphonates tested, namely 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP), 5-amino-1-hydroxypentylidene-1,1-bisphosphonate (AHPeBP) and 6-amino-1-hydroxyhexylidene-1,1-bisphosphonate (AHHexBP). It was about 100 times more active than dichloromethylenebisphosphonate (Cl_2MBP).

Later results where we tested the effect of the bisphosphonate on resorption by its action on the the hypercalcemia induced in thyroparathyroidectomized rats by a synthetic retinoid also showed that AHBuBP was extremely active, inhibiting this increase at a concentration of 0.001 mg P/kg. Also in this test, the AHBuBP was more active than AHPrBP.

Interesting was the result that the local toxicity as measured by necrosis at the site of injection was less for AHBuBP than for AHPrBP. However, it was more than for AHHexBP and AHPeBP, so that it seems that this type of toxicity goes down the longer the side chain.

From these results it would appear that AHBuBP is a very interesting new compound, since it shows a larger efficacy toxicity margin than either AHPrBP or AHHexBP, at least when toxicity is determined by local necrosis. Furthermore, AHBuBP is the strongest compound known up to date in the literature in inhibiting bone resorption. Therefore, I think it is a compound which merits further development in view of an introduction for clinical administration in metabolic bone disease.

You asked me the question whether the effect of this compound could have been foreseen and whether in the future the effect of new bisphosphonates could be foreseen from what we know today. My answer to this question is no. As you can see from the publications I send you enclosed (1-5), we have tested in the past a great variety of bisphosphonates in order to find out a possible structure-effect relationship. And as you see from the results, we have unfortunately not been able to unveil such a relationship. Indeed, an even small change in the side chain can induce a strong alteration of the effect. Furthermore, at least for bone resorption, no correlation could be detected between the effect on crystal dissolution in vitro and the effect on resorption in vivo. With respect to the inhibition of mineralization, a certain correlation has been found in the sense that compounds which do inhibit bone mineralization in vivo, are always inhibitors of apatite crystal formation in vitro. However, the contrary is not always true since even compounds which are good inhibitors of crystal formation in vitro, do not necessarily inhibit bone mineralization in vivo. This lack of any structure-effect relationship with bone resorption led us to the view that the P-C-P part of the compound, if important at all, would only give the compound its bone tropism, while the cellular effect would be due to the side chain. In view of to-day's knowledge it is even not sure that the P-C-P moiety is necessary at all for the inhibition of bone resorption and it could well be that in the future compounds showing another structure than P-C-P but the correct side chain might be effective as well.

Therefore, in my view, and I stressed this repeatedly in my talks on this subject, the bisphosphonates should not be treated as a class of compounds with a specific effect, but the view should be taken that every bisphosphonate is a new compound with its own way of action, its own potency and its own toxicology. This latter point is important for the development of new clinical compounds, since extrapolating toxicological data from one bisphosphonate to another might be totally misleading.

I hope that this information will be of use to you and I remain with kind regards,

Yours sincerely,

H. Fleisch

Prof. H. Fleisch

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Calcif Tissue Int (1983) 35:87–99

Structure-Activity Relationships of Various Bisphosphonates

H. Shinoda[1,*], G. Adamek[1], R. Felix[1], H. Fleisch[1], R. Schenk[2], and P. Hagan[3]

[1]Department of Pathophysiology and [2]Department of Anatomy, University of Berne, Murtenstrasse 35, CH-3010 Berne, Switzerland
[3]Procter & Gamble Company, Cincinnati, Ohio, USA

Summary. A variety of bisphosphonates with aliphatic side chains of increasing length, as well as 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP, formerly APD), dichloromethylene-bisphosphonate (Cl_2MBP, formerly Cl_2MDP), and dibromomethylene bisphosphonate (Br_2MBP, formerly Br_2MDP), were compared in vitro and in vivo to find (a) a possible relationship between structure and activity in order to give some indication about their mechanism(s) of action on bone and (b) the most efficient and safe compound having an effect on bone resorption. Some relationship was found between inhibition of calcium phosphate precipitation in vitro and of mineralization in vivo. No correlation existed, however, between any parameter measured and bone resorption. The number of calvaria cells in culture was decreased by compounds with a chain length greater than 5-C, by AHPrBP, Cl_2MBP, and Br_2MBP. Lactate production by these cells in vitro was increased by the long chain bisphosphonates and AHPrBP, and was decreased by Cl_2MBP. No good correlation existed between the inhibition of bone resorption measured in vitro on calvaria and that seen in vivo on rat tibiae metaphyses. The latter was inhibited the most efficiently by the bisphosphonates longer than 5-C and by AHPrBP; these were 10 times more effective than Cl_2MBP. Taking into account all factors, 1-hydroxypentylidene-1,1-bisphosphonate and AHPrBP seem to be the most active compounds to inhibit bone resorption.

Key words: Bisphosphonates — Mineralization — Bone resorption — Cultured mouse calvaria — Cultured bone cells.

Bisphosphonates are compounds that contain a P-C-P bond and are thus related to pyrophosphate but are resistant to metabolic destruction. They have a strong affinity for calcium phosphate crystals [1] and inhibit both formation [2, 3] and dissolution [4, 5] of this mineral in vitro. In vivo they prevent ectopic calcification and the resorption of bone. Recently 3 bisphosphonates — namely, HEBP, Cl_2MBP, and AHPrBP (formerly EHDP, Cl_2MDP, and APD) (for formula see Table 1) — have been used clinically. HEBP has been found to decrease the development of ectopic ossification after total hip replacement [6] and in paraplegia [7]. Furthermore, HEBP and the other 2 bisphosphonates have proved useful in the management of Paget's disease, a disease in which bone turnover is increased [8–12], and Cl_2MBP and AHPrBP in tumoral bone disease [13, 14].

How the bisphosphonates act in vivo is still speculative. In addition to their physicochemical interaction with calcium phosphate crystals, it was recently found that bisphosphonates also influence cellular metabolism. In cultured cells and/or cultured calvaria HEBP and Cl_2MBP alter glucose metabolism and the oxidation of acetate, citrate, and leucine [15–18]. Cl_2MBP increases the content of cellular glycogen [19], the activity of alkaline phosphatase [20], the oxidation of fatty acids [21], and the synthesis of glycosaminoglycans [22] and of collagen [23].

Many different bisphosphonates can be synthesized by modifying the substituents R_1 and R_2 in the general formula of geminal bisphosphonate (see Table 1). The aims of this work were (a) to study the influence of the chemical structure on the action of the compound and to investigate if a correlation could be found between the effect on the various physicochemical and biochemical processes and the effect on mineralization and bone resorption, giving thus an indication about the mechanism(s) of action in vivo; and (b) to find bisphosphonates with a

* *Present address:* Department of Experimental Pharmacology, Medical Research Institute, Tokyo Medical and Dental University, 3-10, Kandasurugadai, 2-chome Chivoda-Ku, Tokyo, Japan.

Send offprint requests to R. Felix at the above address.

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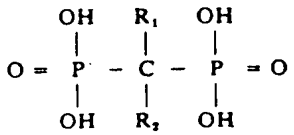
Table 1. List of bisphosphonates studied



Geminal bisphosphonate as acid

Name of compound	Number of C atoms	Abbreviation	R_1	R_2
Methylenebisphosphonate[a]		MBP		
Methanediphosphonate[b]	1	MDP	–H	–H
Hydroxymethylenebisphosphonate[a]		HMBP		
Hydroxymethanediphosphonate[b]	1	HMDP	–OH	–H
1-Hydroxyethylidene-1,1-bisphosphonate[a]		HEBP		
1-Hydroxyethane-1,1-diphosphonate[b]	2	EHDP	–OH	$-CH_3$
1-Hydroxypropylidene-1,1-bisphosphonate[a]		HPrBP		
1-Hydroxypropane-1,1-diphosphonate[b]	3	HPrDP	–OH	$-CH_2CH_3$
1-Hydroxybutylidene-1,1-bisphosphonate[a]		HBBP		
1-Hydroxybutane-1,1-diphosphonate[b]	4	HBDP	–OH	$-(CH_2)_2CH_3$
Pentylidene-1,1-bisphosphonate[a]		PeBP		
Pentane-1,1-diphosphonate[b]	5	PeDP	–H	$-(CH_2)_3CH_3$
1-Hydroxypentylidene-1,1-bisphosphonate[a]		HPeBP		
1-Hydroxypentane-1,1-diphosphonate[b]	5	HPeDP	–OH	$-(CH_2)_3CH_3$
1-Hydroxyoctylidene-1,1-bisphosphonate[a]		HOBP		
1-Hydroxyoctane-1,1-diphosphonate[b]	8	HODP	–OH	$-(CH_2)_6CH_3$
Nonylidene-1,1-bisphosphonate[a]		NBP		
Nonane-1,1-diphosphonate[b]	9	NDP	–H	$-(CH_2)_7CH_3$
1-Hydroxynonylidene-1,1-bisphosphonate[a]		HNBP		
1-Hydroxynonane-1,1-diphosphonate[b]	9	HNDP	–OH	$-(CH_2)_7CH_3$
1-Hydroxydodecylidene-1,1-bisphosphonate[a]		HDBP		
1-Hydroxydodecane-1,1-diphosphonate[b]	12	HDDP	–OH	$-(CH_2)_{10}CH_3$
Dichloromethylenebisphosphonate[a]		Cl_2MBP		
Dichloromethanediphosphonate[b]	1	Cl_2MDP	–Cl	–Cl
Dibromomethylenebisphosphonate[a]		Br_2MBP		
Dibromomethanediphosphonate[b]	1	Br_2MDP	–Br	–Br
3-Amino-1-hydroxypropylidene-1,1-bisphosphonate[a]		AHPrBP		
3-Amino-1-hydroxypropane-1,1-diphosphonate[b]	3	AHPrDP	–OH	$-(CH_2)_2NH_2$

[a] Nomenclature according to IUPAC, *Nomenclature of Organic Chemistry*, Sections A, B, C, D, E, F, and H. Pergamon Press, Oxford, 1979

[b] Old nomenclature

from calvaria tissue into the medium during the incubation was determined as described above.

Resorption was also assessed when the bisphosphonates were given in vivo, as described by Reynolds et al. [26]. The mice received a subcutaneous injection of 1.0 μCi ^{45}Ca on the day of birth. The solutions of bisphosphonates were injected for 3 days (days 4–6) at a volume of 5 μl/g body weight per day. On day 7 the calvaria were dissected and cultured for 48 h. One half was always killed by freezing and thawing 3 times. The cell-mediated ^{45}Ca release for the control and for the treated groups was calculated in percentage as difference between the release of the living and the dead half for each pair of bones (for details see 26).

For the determination of DNA the calvaria were demineralized in 4 ml of 5% (w/v) trichloroacetic acid for 2 days. The residue was then digested for 4 h at 65°C in 2 ml of a solution containing 0.1 M Na-acetate buffer, 5 mM EDTA, 5 mM cystein, pH 5.5, and 1 mg/ml of papain. This resulted in a loss of DNA smaller than 5%. After precipitation by adding 2 ml of 0.5 M $HClO_4$, the DNA was determined according to Burton [27]. DNA was expressed as deoxyribose.

Effect of Bisphosphonates on the Calcification and Resorption of Rat Tibia Metaphysis

Animals. Male or female Wistar rats weighing 160–170 g, in total 220 animals, were used. The rats were bred in this laboratory and maintained on a diet containing 1.1% Ca and 1.2% P (Altromin 1314) and distilled water. The experiments were carried out on animals treated with 16.1 or 161 μmol/kg/day of bisphosphonate given daily subcutaneously for 7 days. For the highly active compounds, the effect of additional doses of 0.16 and 1.61 μmol/kg/day was also tested. The volume injected was kept constant (0.2 ml/100 g body weight). For the dose of 161 μmol/kg/day the bisphosphonates were dissolved in distilled water, and the

Table 2. Influence of bisphosphonates on the minimum formation product [Ca] × [Pi] necessary to induce precipitation of calcium phosphate from solution

Bisphosphonates	Mean increase in formation product from control values in $(mM)^2$ Concentration of bisphosphonates			
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
HEBP	<6.8	<6.8	6.1	1.0
			<7.6	0.6
			<7.1	1.2
HPeBP	<6.8	<6.8	5.6	0.9
			5.2	
HOBP	<6.8	4.8	2.6	0.4
		4.2	2.7	
HNBP	<6.8	3.6	1.0	0.4
		2.8	1.2	
HDBP	3.7	2.3	−1.8	0.3
	3.1	0.3	−1.1	
	1.9		−1.4	
PeBP	<6.8	<6.9	2.4	0.3
			2.3	
			1.6	
NBP	4.1	1.8	1.1	0.5
	3.9	2.4	0.0	0.3
	2.2			
Cl_2MBP	<6.8	2.6	1.2	1.6
		4.6	3.2	0.4
Br_2MBP	<6.9	1.6	0.9	0.0
			1.1	
AHPrBP	<6.9	6.5	5.2	1.6
		6.5	5.0	1.0
			4.3	

The results shown here represent the difference in minimum [Ca] × [Pi] product observed in the presence of the bisphosphonates minus the minimum [Ca] × [Pi] product required for crystal formation in a parallel incubation but in the absence of the bisphosphonate. Control values obtained from 10 separate experiments were 4.37 ± 0.10 (mean ± SEM) in $(mM)^2$. Incubation time was 3 days at pH 7.4 and 37°C. The sign "<" indicates that no precipitation occurred up to this mean increase in formation product

solutions were adjusted to pH 7.4 with NaOH as appropriate. When lower doses were used, the bisphosphonate solutions were diluted with 0.9% (w/v) of NaCl. At least 4 animals were used to test each dose of each compound. During the experiment, all the animals had free access to food and drinking water. One day before the beginning of the treatment with bisphosphonate, each animal received oxytetracycline 15 mg/kg body weight. This fluorescent compound localizes on the newly formed bone and can therefore be used as a marker for the longitudinal growth of the tibia (see below). At the end of the experiment the rats were killed with ether.

Histological Techniques. The proximal ends of the tibiae were removed from the animals, cleaned of the attached muscles and soft tissues, and fixed in 40% precooled ethanol (4–6°C) for more than 24 h. After blockstaining and dehydration in graded ethanol (70–100%) containing 0.25% basic fuchsin and clearing in xylene, the specimens were embedded in methylmethacrylate. Undecalcified, 80 μm thick ground sections were examined by fluorescence microscopy and microradiography. Measurements

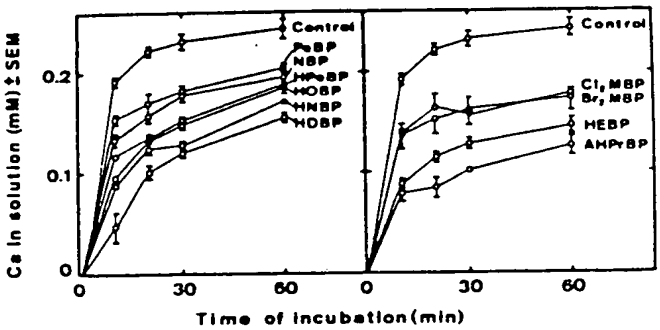


Fig. 1. Effect of bisphosphonates on the dissolution of hydroxyapatite crystals in vitro. Crystals, which had been treated with bisphosphonates (0.075 mol/mol of apatite), were added to buffer free of bisphosphonate. The dissolution was followed by measuring the calcium concentration at various times. For more details see [5]. The mean ± SEM of 3–6 experiments is given. For symbols see Table 1

of longitudinal growth and width of the epiphyseal plates in the proximal end of the tibiae were performed under the fluorescent microscope at a magnification of 25×. The distance between the epiphyseal borderline of the growth cartilage and the most proximal tetracycline label in the metaphysis was determined [28]. X-ray micrographs were used to locate the calcification front in the growth cartilage and to estimate the density of the mineralized trabecular bone in the metaphysis.

Statistics

The values are given as mean ± SEM of N dishes. Significance of difference was determined according to the Student's t test. When pairs of calvaria halves were compared, pair analysis was made.

Results

Effect of Bisphosphonates on Mineral Formation

As shown in Table 2, all the bisphosphonates tested inhibited mineral formation. The effect decreased with increasing length of the aliphatic side chain. Interestingly, HDBP at 10^{-6}M stimulated the precipitation of calcium phosphate. Br_2MBP was somewhat less active than Cl_2MBP.

Effect of Bisphosphonates on the Dissolution of Apatite

Figure 1 shows that when apatite was treated with bisphosphonates, the mineral dissolved more slowly than in the controls. In this figure only the values for the dissolved Ca^{2+} are given; the data for dissolved Pi were similar (not shown). Of all the compounds tested, AHPrBP and HEBP showed the

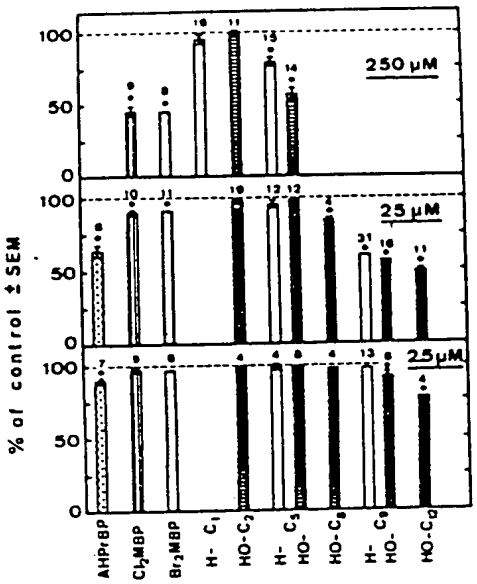


Fig. 2. Effect of bisphosphonates on cell number. 100,000 cells were plated in 24-well cluster dishes. The bisphosphonates, 250, 25, and 2.5 μM, were present from day 1 until the end of the experiment (day 8). The cell number in the controls was 611, 400 ± 9800 (119) (mean ± SEM [N]). For symbols see Table 1. The number above the bars is N. * significantly different from control. $P < 0.001$

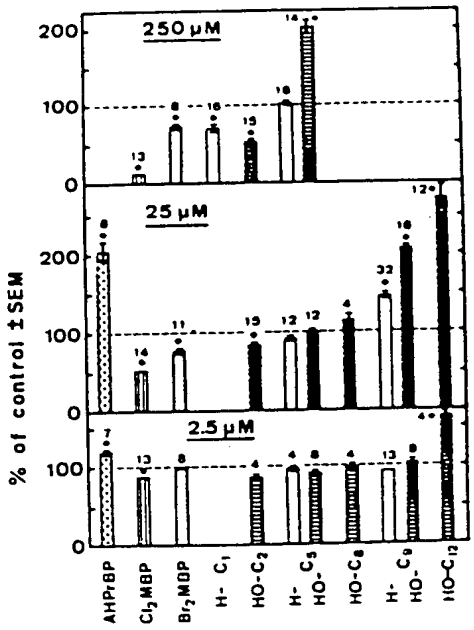


Fig. 3. Effect of bisphosphonates on the lactate production of rat calvaria cells. Lactate production was measured from day 7 to 8 (16 h). In the control the calvaria cells produced 7.98 ± 0.12 μmol of lactate/10^6 cells (119) (mean ± SEM [N]). For details see Fig. 2. * significantly different from control, $P < 0.001$

strongest effect. Results from the left side of Fig. 1 indicate that an increase in the length of the aliphatic side chain as well as the presence of a hydroxyl group (R_1) strengthened the effect of these compounds. Br_2MBP was not different from Cl_2MBP.

Effect of Bisphosphonates on Calvaria Cells in Culture. Cell Number. Figure 2 shows the effect on the cell number. Bisphosphonates with a long side chain decreased the cell number more than bisphosphonates with a short side chain. No cells remained at all in the presence of 250 μM bisphosphonates with 8, 9, and 12 C atoms and also in the presence of 250 μM AHPrBP. The presence of the hydroxyl group (R_1) caused a slight decrease in the cell number. It should be noted that AHPrBP and HDBP at 250 μM precipitated with calcium. Cl_2MBP and Br_2MBP decreased the cell number also, but less than the bisphosphonates mentioned above.

Lactate. As shown in Fig. 3, the bisphosphonates with a long side chain increased lactate production, those with a short chain decreased it. The change seems to be with PeBP. Interestingly, as observed for the cell number, AHPrBP behaved like a bisphosphonate with a long side chain, strongly increasing lactate production. Compounds with a hydroxyl group (R_1) instead of −H augmented lactate production more. From all the compounds tested, Cl_2MBP decreased lactate production to the greatest extent; Br_2MBP, however, influenced this parameter much less than Cl_2MBP. Glucose consumption was also measured in the cells treated with MBP, Cl_2MBP, PeBP, NBP, HDBP, and AHPrBP, and was found to be affected in a parallel way to lactate production (not shown).

Table 3. Effects of bisphosphonates on the K^+ content of cultured calvaria cells

Bisphosphonate (25 μM)	% of control ± SEM (N)
Control	100.0 ± 1.2 (23)
PeBP	123.1 ± 4.2 (8)[b]
HPeBP	99.6 ± 1.8 (8)
NBP	101.1 ± 2.6 (17)
HNBP	120.1 ± 4.3 (15)[b]
HDBP	100.5 ± 9.1 (8)
Cl_2MBP	106.8 ± 2.6 (16)[a]
AHPrBP	98.9 ± 6.9 (17)

The cells were cultured in dishes with a diameter of 3.5 cm in the presence or absence of bisphosphonate from day 1 to 7. The K^+ content of the control cells was 0.170 ± 0.008 (23) μmol/10^6 cells
[a] Significantly different from control, $P < 0.05$; [b] $P < 0.001$

Potassium. K^+ content was not influenced by most of the bisphosphonates. There was some increase in the cells treated with PeBP and HNBP (Table 3).

Table 4. Effects of bisphosphonates on the alkaline phosphatase activity of cultured calvaria cells

Bisphosphonates (25 μM)	% of control ± SEM (*N*)
Control	100.0 ± 7.1 (5)
PeBP	110.6 ± 5.8 (4)
HPeBP	115.3 ± 3.5 (4)
HBP	128.2 ± 2.4 (4)[a]
HNBP	57.6 ± 4.7 (4)[a]
HDBP	50.6 ± 9.4 (4)[a]
Cl_2MBP	217.6 ± 3.5 (4)[b]
AHPrBP	57.6 ± 2.4 (4)[b]

The cells were cultured in 24-well cluster dishes in the presence or absence of bisphosphonate from day 1 to 7. The enzyme activity of the control was 8.5 ± 0.6 (5) nmol/min/10^6 cells
[a] Significantly different from control, $P < 0.05$; [b] $P < 0.01$

Table 5. Effects of Cl_2MBP and Br_2MBP on the alkaline phosphatase activity

Bisphosphonates	Alkaline phosphatase activity % of control ± SEM (*N*)
Control	100.0 ± 2.2 (16)
Cl_2MBP (μM)	
250	634.5 ± 33.3 (8)[b]
25	207.5 ± 9.5 (8)[b]
2.5	113.4 ± 6.8 (8)[a]
Br_2MBP (μM)	
250	208.9 ± 9.0 (8)[b]
25	144.6 ± 7.7 (8)[b]
2.5	99.0 ± 5.0 (8)

For details see Table 4. The activity of the control was 13.05 ± 0.54 (16) nmol/min/10^6 cells
[a] Significantly different from control, $P < 0.05$; [b] $P < 0.001$

Alkaline Phosphatase. As shown in Tables 4 and 5, the activity of alkaline phosphatase in calvaria cells was decreased by HNBP, HDBP, and AHPrBP, not affected by PeBP or HPeBP, slightly increased by NBP, and strongly enhanced by Br_2MBP and especially by Cl_2MBP. Cl_2MBP, but not Br_2MBP, was still active at 2.5 μM.

Effects of Bisphosphonates Given in Vitro on Cultured Mice Calvaria

As shown in Figs. 4 and 5, all the bisphosphonates except HDBP inhibited the release of ^{45}Ca. The effect was smaller the longer the aliphatic side chain. Compounds with a hydroxyl group (R_1) acted somewhat more weakly, but not significantly.

The compounds containing up to 5 C atoms inhibited lactate production, except AHPrBP which had no effect; compounds with more than 5 C atoms, on the other hand, increased it (Fig. 4). NBP and Cl_2MBP exerted an influence at the concentration of 25 μM (Fig. 6).

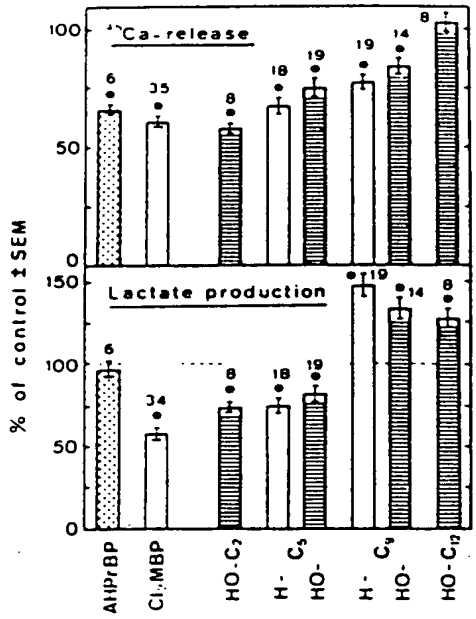


Fig. 4. Effect of 250 μM bisphosphonates on ^{45}Ca release and lactate production of mice calvaria in culture. The 2 halves of a calvarium were cultured for 48 h, respectively, in the presence or absence of the bisphosphonate. The ^{45}Ca content in the medium as a percentage of the total (bone and medium) was 19.4 ± 0.4 (127) % (mean ± SEM [*N*]) for the control half-calvaria. The lactate production was 2.46 ± 0.01 (126) μmol (mean ± SEM [*N*]). The number above the bar represents the number of half-calvaria. For symbols see Table 1. * significantly different from control, $P < 0.005$

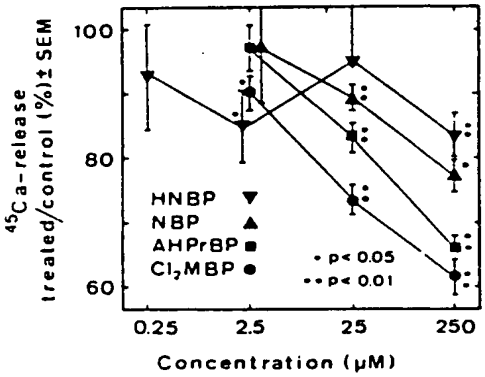


Fig. 5. Effect of various bisphosphonates added in vitro on the ^{45}Ca release of mice calvaria in culture. For details see legend of Fig. 4. Between 5 and 22 half-calvaria were tested. Statistically significant difference vs control: *$P < 0.05$; **$P < 0.01$

Effects of Bisphosphonates Given in Vivo on Cultured Mice Calvaria

Cl_2MBP or HNBP was injected into young mice and the calvaria then cultured for 48 h. As shown in Fig.

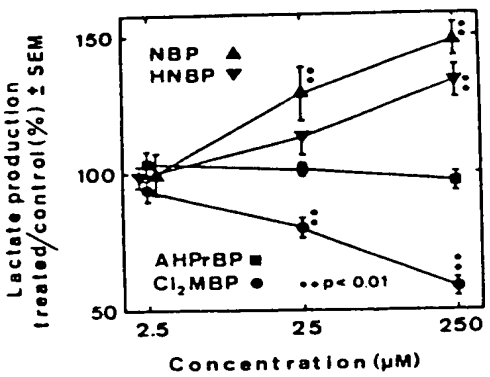


Fig. 6. Effects of Cl_2MBP, AHPrBP, NBP, and HNBP added in vitro on the lactate production of mice calvaria in culture. For details see Fig. 5

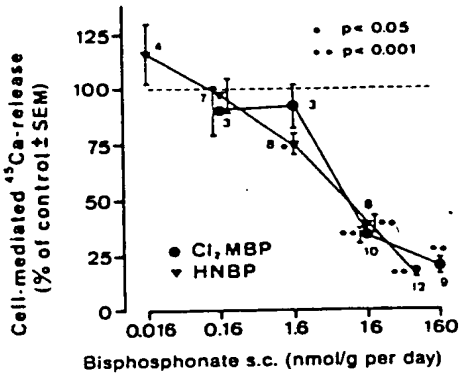


Fig. 7. Effects of Cl_2MBP and HNBP given in vivo on the ^{45}Ca release of mice calvaria in culture. The mice were treated from days 4 to 6 with the bisphosphonate. On day 7 half-calvaria were cultured for 48 h in absence of the bisphosphonate. The cell-mediated ^{45}Ca release is calculated in % as difference between the release of the living and the dead half for each pair of bones. Cell-mediated ^{45}Ca release of the control was 10.9 ± 0.5 (N = 39) %. Number of half-calvaria is given at each point. Statistically significant difference vs control, $^{*}P < 0.05$; $^{**}P < 0.001$

7, both compounds inhibited the release of ^{45}Ca to the same degree. At the highest concentration (161 nmol/g/day) Cl_2MBP inhibited lactate production (Table 6), but it had no effect at 10 times lower concentrations (not shown). HNBP given at 80.5 nmol/g/day (at 161 nmol/g the animals died) decreased DNA content, but lactate production, normalized per amount of DNA, was not affected (Table 6). Lower concentrations of HNBP showed no effect (not shown).

Effects of Bisphosphonates Given in Vivo on Resorption of Metaphyseal Cartilage and Bone Epiphyseal Plate and Mineralization in Growing Rats

Bone and calcified cartilage resorption were evaluated semiquantitatively in contact microradiographs of the proximal end of the tibiae in rats (Fig. 8). Rating of resorption inhibition is based on the density and structure of the metaphyseal cancellous bone formed during the observation period. This accretion zone can be delineated by the tetracycline label given before treatment, which separates it from the remodeling zone present before bisphosphonate administration. In controls (Fig. 8A) the accretion zone presents a characteristic microradiographic pattern. Its proximal third consists of an array of fine, densely packed mineralized septa representing the longitudinal calcified cartilage (walls) surrounding the hypertrophic cell columns. More distally these calcified septa are reduced in number but increased in diameter by deposition of mineralizing bone on their surface. This process continues further distally into the remodeling zone and finally leads to a substitution of the primary spongiosa (still containing remnants of calcified cartilage) by the secondary spongiosa. The numerical reduction and remodeling require a considerable amount of bone and calcified cartilage resorption by osteoclasts and chondroclasts. Partial or complete suppression of the activity of these cells must result in a marked increase in density of the mineralized tissue, especially in the accretion zone. Thus the numerical reduction of trabeculae (equal to a widening of the intertrabecular spaces) is less pronounced (Fig. 8B, C) or completely absent (Fig. 8D). Furthermore, a complete arrest of bone resorption also results in more cylindrical shape of the metaphysis in the accretion zone. In the examples shown in Fig. 8, C is rated as strong inhibition (++), D as very strong (+++). A delayed onset of these changes, indicated by their position relative to the tetracycline label, is considered as a slight inhibition (+). Finally, if a slight inhibition was not uniformly observed in all animals of one group, this is rated as questionable (±).

As shown in Table 7, AHPrBP, HPeBP, and HOBP were the most powerful inhibitors of bone resorption with a marked effect at a dose of 1.6 μmol/kg/day. They are followed by NBP and HNBP and then by Cl_2MBP, Br_2MBP, and HDBP, which are strongly active at 16.1 μmol/kg. Many of the compounds inhibit mineralization at 161 μmol/kg, namely, HEBP, HPrBP, HBBP, HPeBP, and AHPrBP (Table 8). Interestingly, neither Cl_2MBP, Br_2MBP, nor NBP showed such an effect. No compound showed an inhibition of mineralization at 16.1 μmol/kg. The longitudinal growth was inhibited by AHPrBP, HNBP, NBP, HOBP, HPeBP, HPrBP, Cl_2MBP, and HEBP at 161 or 16.1 μmol/kg. This might be due, at least partially, to the strong inhibition of the resorption of the calcified cartilage,

Table 6. Effects of Cl_2MBP and HNBP, given in vivo, on the lactate production of mice calvaria cultured in vitro

	N	Lactate (μmol)	Desoxyribose (nmol)	Lactate/desoxyribose (μmol/nmol)
Control	13	4.93 ± 0.36	7.92 ± 0.53	0.65 ± 0.06
		(%)	(%)	(%)
Control	13	100.0 ± 6.5	100.0 ± 3.1	100.0 ± 5.3
Cl_2MBP	15	64.0 ± 3.2[b]	97.4 ± 2.4	65.8 ± 2.5[b]
		(μmol)	(nmol)	(μmol/nmol)
Control	7	5.15 ± 0.41	9.59 ± 0.30	0.54 ± 0.04
		(%)	(%)	(%)
Control	7	100.0 ± 4.7	100.0 ± 2.7	100.0 ± 5.0
HNBP	7	82.1 ± 6.3[a]	71.7 ± 4.2[b]	114.7 ± 9.1

Cl_2MBP (161 nmol/g/day) or HNBP (80.5 nmol/g/day) was injected from days 4 to 6 subcutaneously. The calvaria were cultured from day 7 for 48 h in the absence of bisphosphonate. The results are given as mean ± SEM of N half-calvaria

[a] Significantly different from control, $P < 0.05$

[b] Significantly different from control, $P < 0.001$

diminishing vascular invasion. HDBP, HNBP, NBP, AHPrBP, and HEBP impaired increase in body weight. Some of the long-chain compounds — HOBP, HNBP, and NBP — were toxic at the highest dose, the animals dying during treatment.

Discussion

The investigation of these bisphosphonates can be subdivided in a physicochemical part (formation and dissolution of mineral), a part in which the effects on cultured cells and cultured calvaria were studied, and a part in which the effects in vivo (resorption and mineralization) were examined.

HEBP, HPeBP, and AHPrBP were the strongest inhibitors of mineral formation in vitro. The inhibition decreased with increasing chain length: this could be the result of an increasing hydrophobic nature of the side chain diminishing the affinity of the bisphosphonate for apatite. The hydroxyl group at C-1, on the other hand, seems to increase slightly the inhibitory action of the compounds. Interestingly, HDBP slightly stimulated crystal formation at 10^{-6} M. At this concentration HDBP induced a precipitation in a solution of 1.7 mM $CaCl_2$, free of Pi. This precipitate, probably of Ca-HDBP, possibly acted as a nucleator of calcium phosphate crystallization.

The dissolution of apatite was inhibited by all the bisphosphonates to some degree, AHPrBP and HEBP being the most active. There is no strong relationship between the structure of these bisphosphonates and their effect.

In cultivated cells, in contrast to bone culture, no mineral is present. Any actions of bisphosphonates observed in this system are therefore direct effects on the cells and cannot be secondary effects due to physicochemical interactions with calcium phosphate crystals. It had been shown earlier that Cl_2MBP, but not HEBP, at a concentration of 0.25 mM diminished the cell number, and that both bisphosphonates decreased glycolysis [15]. This was also observed in this study. Br_2MBP acted more weakly than Cl_2MBP. Bisphosphonates containing more than 5 C atoms decreased cell number, the activity increasing with increasing length of the side chain. At a concentration of 250 μM, all cells died. In contrast to that in the short-chain bisphosphonates, the lactate production was increased by these compounds, activity increasing again with increasing length. Interestingly, AHPrBP, which contains only 3 C atoms but an amino group at C-3, acted similarly to the long-chain bisphosphonates. Since AHPrBP and HDBP precipitated with calcium at 250 μM, it might be that this augmented the effect of these compounds at this concentration. It has been shown that bisphosphonates, added to macrophages, are more cytotoxic in the presence of bone mineral than when added in solution [29]. The reason might be that the cells engulf the crystals and ingest therefore a larger amount of bisphosphonate. It is not unexpected that long-chain bisphosphonates are cytotoxic at high concentrations, since all these compounds contain a lipophilic side chain and negatively charged phosphonate groups. They might act as detergents and damage the cell membrane. It is possible that the higher permeability of the cell membrane increases the intracellular ratio of Ca^{2+} to Mg^{2+}. Such a higher ratio and a resulting higher lactate production has been observed in ascites tumor cells after adding ionophore A 23187 [30]. Another possibility is that the long-chain bisphosphonates act as uncouplers of oxidative phosphorylation, as do long-chain fatty acids, which would also stimulate glycolysis. This last interpre-

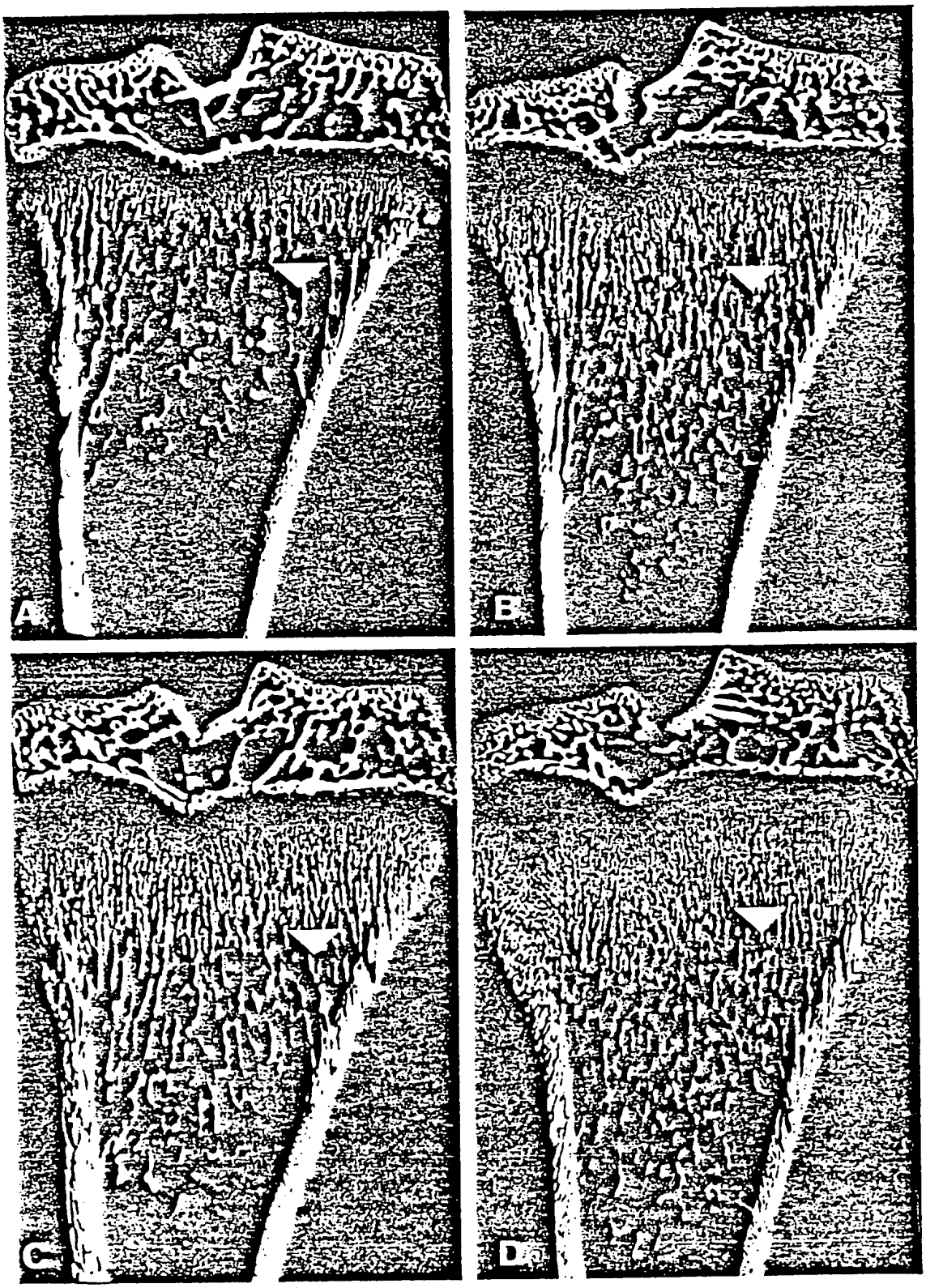


Fig. 8. Criteria for the evaluation of resorption inhibition in vivo. Contact microradiographs of the proximal tibia in rats. ×10. A NaCl control. B HNBP 1.61 (rated +). C HPeBP 1.61 (rated ++). D HPeBP 16.1 (rated +++). The position of the most proximal tetracycline label given at the onset of treatment is indicated by the baseline of the white triangles

Table 7. Effects of 7 days of bisphosphonate treatment on resorption in epiphysis and metaphysis of tibiae in growing rats

Bisphosphonates dose: (μmol/kg)	Inhibition of bone resorption			
	0.16	1.61	16.1	161
MBP				++
HMBP				+
HEBP			+	+
HPrBP			±	±
HBBP			−	±
PeBP			±	+++
HPeBP	−	++	+++	++
HOBP	±	++	+++	died
NBP	−	±	+++	++
HNBP	−	+	+++	died
HDBP			++	
Cl_2MBP			++	+++
Br_2MBP			++	+++
AHPrBP	−	++	+++	++

Qualitative evaluation was made on the contact microradiographs of tibial ground sections. −, no inhibition; ±, questionable; +, slight inhibition; ++, strong inhibition; +++, very strong inhibition

tation, however, is unlikely since, when untreated cells were incubated in the absence of oxygen, glycolysis was increased by only about 40% (Felix and Fleisch, unpublished observations), while bisphosphonate treatment resulted in an increase of up to 175%. The mechanism by which HEBP, Cl_2MBP, and Br_2MBP act on the cells is unknown and needs further study. Results obtained in vitro must be extrapolated with caution. Relatively high concentrations were used. The concentrations in vivo, however, are not known and might also be high around or inside a cell resorbing bone mineral coated with bisphosphonates.

Alkaline phosphatase has been shown to be increased in cultured calvaria cells by Cl_2MBP [20]. A molecule containing halogen atoms seems to be essential for this action in cultured cells, since Br_2MBP was the only other bisphosphonate that showed such an effect, although weaker than that of Cl_2MBP. Increased alkaline phosphatase activity was also found in rat bone [31] and in epiphyseal cartilage of chicken [32] after administration of HEBP or Cl_2MBP in vivo. The significance of this observation is unknown. Other compounds, among them cortisol analogs, are known to increase alkaline phosphatase in cultured cells too (for references see 20).

When bisphosphonates are added to cultivated calvaria, their effect on lactate production is similar to that in cultivated cells, long-chain bisphosphonates increasing, short-chain decreasing the production, with the exception of AHPrBP, which had no effect on lactate production in calvaria.

Some discrepancies exist between the results with calvaria when the bisphosphonates are given in vitro and in vivo. Thus HNBP increased lactate production only when applied in vitro, but not when applied in vivo. Similarly, when HNBP was added in vitro, the effect on Ca release was weaker than that of Cl_2MBP, whereas the two compounds were similar when they were given in vivo but tested in vitro.

It was hoped that the comparison of the various parameters thought to be involved in mineralization and bone resorption, and the results seen with the various bisphosphonates (Table 9), would give some insight into the mechanisms inducing these processes and the mode of action of the bisphosphonates. This seems to be so to a certain extent for mineralization. Indeed, compounds that were potent inhibitors of crystal formation also inhibited mineralization in vivo. This suggests that the effect in vivo might, at least partly, be due to physicochemical interaction with the mineral. However, other actions of the bisphosphonates cannot be excluded. It has also been found that HEBP induces the accumulation of proteoglycan superaggregates in the epiphyseal growth plate; these superaggregates are characterized by their capacity to inhibit the crystallization of calcium phosphate [33, 34].

When considering bone resorption the results are less encouraging. Thus there was no correlation between the inhibition of bone resorption in vivo or in calvaria and the inhibition of mineral dissolution, which suggests that the bisphosphonates inhibit bone resorption not by their physicochemical interaction alone. Furthermore, bisphosphonates did not have the same effect on bone resorption when they were injected into growing rats or when they were studied in cultured mice calvaria. The cause of this discrepancy is not clear. A different distribution to and within the bone in the two conditions is a possibility. That this discrepancy is due to species difference is unlikely. Since Cl_2MBP has been shown to decrease lactate production more than HEBP [15], and since formation of lactic acid might be involved in bone resorption [35], it has been suggested that the bisphosphonates might act by this metabolic effect [15]. The results, however, obtained with the long-chain bisphosphonates do not support such a mechanism, since they are strong inhibitors of bone resorption, although they increase, rather than decrease, lactate production. This agrees with recent data which suggest that lactate production does not play a major role in bone resorption [36–38]. Some correlation is seen, however, between the toxicity of the compounds on the cells and resorption, so that this mechanism could be important.

Our interpretation is in disagreement with that of

Table 8. Effects of bisphosphonates on mineralization and growth of tibial epiphysis and on increase of body weight of growing rats

Bisphosphonate	Dose μmol/kg/day	Epiphyseal width	Longitudinal growth	Increase of body weight
HEBP	161	288.3 ± 7.8 (17)[c]	84.7 ± 2.4 (16)[c]	35.9 ± 8.7 (25)[c]
HEBP	16.1	118.2 ± 5.3 (4)	107.8 ± 1.7 (4)	118.6 ± 8.1 (4)
HPrBP	161	290.9 ± 30.6 (4)[c]	69.1 ± 6.3 (4)[b]	91.5 ± 10.5 (4)
HBBP	161	349.6 ± 8.7 (4)[c]	90.9 ± 5.4 (4)	82.5 ± 6.5 (4)
HBBP	16.1	90.8 ± 7.5 (4)	95.4 ± 3.7 (4)	99.0 ± 11.5 (4)
PeBP	161	88.3 ± 5.7 (6)	99.7 ± 3.2 (5)	61.3 ± 12.9 (4)
PeBP	16.1	87.8 ± 3.9 (6)[a]	103.4 ± 3.9 (5)	72.4 ± 7.9 (4)
HPeBP	161	235.8 ± 17.7 (4)[c]	72.1 ± 2.7 (4)[a]	75.3 ± 3.6 (4)
HPeBP	16.1	68.0 ± 4.1 (4)[b]	85.2 ± 2.7 (4)	97.3 ± 3.6 (4)
HPeBP	1.6	89.6 ± 5.3 (4)	98.8 ± 2.9 (4)	108.5 ± 12.5 (4)
HOBP	161		animals died	
HOBP	16.1	68.0 ± 4.1 (4)[b]	77.1 ± 3.1 (4)[a]	93.7 ± 2.2 (4)
HOBP	1.6	100.0 ± 9.2 (4)	98.3 ± 7.1 (4)	108.5 ± 7.1 (4)
NBP	161	129.7 ± 14.2 (7)	61.5 ± 5.2 (7)[c]	half of the animals died
NBP	16.1	77.3 ± 1.1 (9)[c]	90.7 ± 2.4 (8)[a]	89.1 ± 3.4 (4)[a]
NBP	1.6	97.1 ± 3.1 (8)	94.2 ± 1.7 (8)	86.8 ± 5.6 (8)[a]
HNBP	161		animals died	
HNBP	16.1	64.0 ± 6.5 (4)[b]	81.7 ± 4.4 (4)	52.9 ± 9.0 (4)[a]
HNBP	1.6	95.4 ± 8.7 (4)	100.0 ± 17.2 (2)	109.5 ± 13.6 (4)
HDBP	16.1	101.9 ± 13.2 (4)	114.6 ± 7.2 (4)	21.3 ± 13.4 (6)[a]
Cl_2MBP	161	72.8 ± 3.2 (14)[c]	80.5 ± 3.4 (14)[c]	103.0 ± 8.4 (22)
Cl_2MBP	16.1	80.5 ± 2.8 (19)[c]	90.4 ± 3.6 (17)[a]	99.2 ± 9.4 (31)
Br_2MBP	161	118.6 ± 13.4 (5)	71.3 ± 7.8 (3)[a]	83.6 ± 6.5 (5)
Br_2MBP	16.1	91.9 ± 4.6 (3)	91.7 ± 5.1 (3)	98.0 ± 27.8 (3)
AHPrBP	161	225.1 ± 4.9 (4)[c]	66.1 ± 2.0 (4)[c]	32.2 ± 12.4 (4)[b]
AHPrBP	16.1	70.9 ± 4.2 (4)[a]	85.8 ± 6.1 (3)[a]	100.0 ± 6.2 (4)
AHPrBP	1.6	83.3 ± 6.9 (4)	96.5 ± 3.0 (4)	103.1 ± 5.3 (4)

The results are presented as percentage of the control, since the absolute values of the control varied from experiment to experiment. The absolute mean values of the control were for the epiphyseal width, 423 ± 11 (47) μm; for the longitudinal growth of epiphysis, 2193 ± 56 (46) μm; and for increase of body weight, 24 ± 2 (75) g (mean + SEM [N])

[a] Significantly different from control, $P < 0.05$

[b] Significantly different from control, $P < 0.005$

[c] Significantly different from control, $P < 0.001$

Table 9. Summary of the inhibitory effects of the bisphosphonates in the different systems

				Calvaria cells		Cultured calvaria		Bone	
	R_1	Crystal formation	Crystal dissolution	Cell number	Lactate production	Lactate production	^{45}Ca release	resorption in vivo	Mineralization in vivo
MBP	−H	3[a]	2[b]	0	1	−	−	1	1
HMBP	−OH	3[a]	2[b]	−	−	−	−	1	1
HEBP	−OH	3	2	0	2	1	3	2	1
PeBP	−H	3	1	1	0	1	3	1	0
HPeBP	−OH	3	1	1	1(↑)	1	2	3	1
HOBP	−OH	2	1	3	1(↑)	-	−	3	(died)
NBP	−H	1	1	3	2(↑)	1(↑)	2	2	0
HNBP	−OH	2	2	3	2(↑)	1(↑)	1	3	(died)
HDBP	−OH	1	2	3	3(↑)	1(↑)	0	2	−
Cl_2MBP	−Cl	2	1	2	3	2	3	2	0
Br_2MBP	−Br	2	1	2	1	−	−	1	0
AHPrBP	−OH	3	3	3	2(↑)	0	3	3	1

For the first 6 columns the symbols mean: 0, no effect; 1, weak effect; 2, medium effect; 3, strong effect; −, not done. Some bisphosphonates increased the lactate production compared to the control; this is indicated by (↑). For the 7th column the grading was the following: 3, the lowest dose having an effect on resorption was 1.6 μmol/kg/day; 2, it was 16.1 μmol/kg/day; 1, it was 161 μmol/kg/day; 0, no effect was observed. In the last column, 1 means that an inhibition of mineralization occurred at 161 μmol/kg; 0, no inhibition was seen

[a] Taken from Fleisch et al. [3]

[b] Taken from Russell et al. [5]

Reitsma et al. [39] proposed in their study of AHPrBP. Since inhibition of bone resorption occurred at lower concentration than mineralization, they suggested that inhibition of bone resorption would mainly be due to the physicochemical stabilization of bone mineral, whereas the inhibition of mineralization might be due to a toxic effect on bone cells.

Whatever the final answer, it has to be kept in mind that more than one mode of action may be involved, so that crystal dissolution, lactic acid production, and cell toxicity could all play a role.

Looking for the most favorable drug against diseases involving increased bone resorption, Cl_2MBP, Br_2MBP, HPeBP, and AHPrBP seem to be the best candidates, whereby the effects of Cl_2MBP and Br_2MBP are very similar. Cl_2MBP and Br_2MBP do not inhibit mineralization, in contradiction to HPeBP and AHPrBP, which inhibit mineralization at 161 μmol/kg/day. These two compounds are, however, at least 10 times more potent inhibitors of bone resorption than Cl_2MBP and Br_2MBP, inhibiting bone resorption at a dose 100 times lower than the one necessary to inhibit mineralization. HPeBP has about a 10 times weaker cell toxicity than AHPrBP. The choice for use in human diseases will be made by their effect on the specific disease involved and by the type and number of possible side effects.

The bisphosphonates showing strong toxic effect on cultivated cells might be envisaged as useful therapy for neoplastic bone diseases. Since these compounds accumulate only in bone, they would act specifically at this location.

Finally, these results show that the various bisphosphonates vary a great deal in their respective effects. This implies that every one of them has to be considered as a compound per se, their structure of bisphosphonates only making them go specifically to bone and other mineralized tissues.

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Effect of Various Polyphosphonates on Ectopic Calcification and Bone Resorption in Rats

Roman C. Mühlbauer, Herbert Fleisch

Department of Pathophysiology, University of Berne, Switzerland

Key Words. Polyphosphonates · Bone resorption · Ectopic calcification · Calcemia · Parathyroid hormone · Rats

Abstract. Nine new polyphosphonates have been tested and compared with dichloromethane diphosphonate (Cl_2MDP) as to their inhibitory effect on soft tissue calcification and on bone resorption. The geminal diphosphonates (P-C-P) were found to be most active in inhibiting aortic and renal calcification induced by vitamin D_3 intoxication. Only the compounds with an elongated carbon chain matched Cl_2MDP in activity. Similar results were obtained for bone resorption tested on the hypercalcemia induced by parathyroid hormone in thyroparathyroidectomized animals. Again only the long-chain diphosphonates matched Cl_2MDP. These results suggest that changes in chain length may prove successful in the search for more effective diphosphonates in the future.

Introduction

Polyphosphonates, compounds similar in structure to polyphosphate, but characterized by P-C bonds, have received increased attention this last decade. They were found to have strong effects both on calcification and bone resorption. Most of the compounds tested were geminal diphosphonates which are characterized by a P-C-P structure. Some of these were found to inhibit very efficiently ectopic [1], as well as bone and cartilage calcification [2–4] when given orally or parenterally. This effect is explained by their ability to inhibit the formation of calcium phosphate crystals [1, 5]. Furthermore, some of the geminal diphosphonates also inhibit bone resorption both when assessed in organ culture [6, 7] and in vivo [2, 6].

These various characteristics have been utilized clinically. Thus, 1-hydroxyethane-1,1-diphosphonate (EHDP) has been shown to inhibit ectopic bone formation in patients with paraplegia [8] and with hip replacements [9]. Furthermore, it decreases bone turnover in Paget's disease [10, 11]. Cl_2MDP has recently been shown to be also very efficient in reducing bone turnover in Paget's disease [12]

Table I. List of polyphosphonates studied

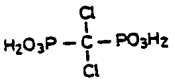
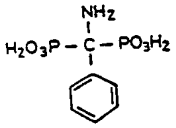
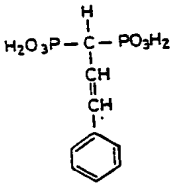
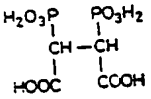
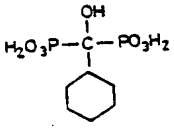
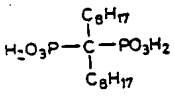
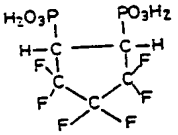
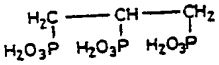
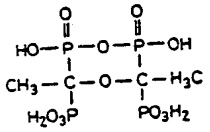
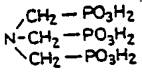
Name of compound	Abbreviation	Formula
First series of experiments		
Dichloromethane diphosphonic acid	Cl_2MDP	
Phenylaminomethane diphosphonic acid	PAMDP	
3-Phenylprop-2-ene-1,1-diphosphonic acid	PPDP	
1,2-Dicarboxyethane-1,2-diphosphonic acid	DCEDP	
Cyclohexylhydroxymethane diphosphonic acid	CHMDP	
Heptadecane-9,9-diphosphonic acid	HDDP	
Second series of experiments		
3,4,5-Hexafluorocyclopentane-1,2-diphosphonic acid	HFCPDP	
Propane-1,2,3-triphosphonic acid	PTP	
Condensate II of ethane-1-hydroxy-1,1-diphosphonic acid	condensate II	
N,N,N-Triphosphonomethylamine	TPMA	

Table II. Influence of various polyphosphonates at two doses on vitamin D_3-induced calcification of aortas and kidneys; calcium content is given as µg Ca/mg dry tissue ± 1 SEM (number of determinations in parentheses)

Compound	Aorta				
	1 mg P/kg BW s.c.			10 mg P/kg BW s.c.	
	survival			survival	
Experiment 1					
Cl_2MDP	7/10	2.93± 0.98[1]	(7)	10/10	2.49± 1.00[1] (10)
PAMDP	10/10	11.85± 3.81[3]	(8)	9/10	3.19± 0.90[1] (8)
PPDP	8/10	11.52± 5.19[2]	(8)	9/10	1.85± 0.40[1] (8)
DCEDP	10/10	54.61±17.05	(10)	5/10	11.56± 8.54 (3)
CHMDP	10/10	15.28± 5.77[3]	(9)	6/10	6.75± 3.05[3] (4)
HDDP	8/10	3.01± 1.06[1]	(7)	0/10	
No vitamin D_3	15/15	1.86± 0.51	(14)	significant difference $\alpha < 0.002$	
Vitamin D_3 alone	41/46	60.5 ± 7.86	(39)		
Experiment 2					
Cl_2MDP	10/10	5.09± 2.42[3]	(10)	9/10	10.51± 7.46 (9)
HFCPDP	9/10	8.19± 4.05[3]	(9)	9/10	25.21± 7.88 (9)
PTP	9/10	64.37±23.20	(8)	10/10	21.50±10.05 (8)
Condensate II	10/10	25.60±11.38	(10)	10/10	29.36± 8.48 (10)
TPMA	10/10	38.85±15.0	(9)	10/10	6.45± 2.69[3] (10)
No vitamin D_3	9/10	1.51± 0.40	(8)	significant difference $\alpha < 0.002$	
Vitamin D_3 alone	34/35	40.29± 8.31	(32)		

[1] The difference between treatment mean and mean of vitamin D_3 alone is significant, $\alpha < 0.002$.
[2] $\alpha < 0.01$.
[3] $\alpha < 0.05$.

and bone resorption in tumoral bone disease [13]. Finally, 3-amino,-1-hydroxypropane-1,1-diphosphonate has also been found to be active in Paget's disease [14] and against tumoral hypercalcemia [15].

The results obtained so far indicate that biological effects are modified by small molecular changes. It is by no means sure that the compounds used today clinically are the most efficient ones. We have, therefore, investigated in this study a series of new polyphosphonates with respect to their effect on ectopic calcification and bone resorption.

Methods and Materials

Prevention of Aortic and Kidney Calcification Induced by Vitamin D_3 in Rats

The experimental design was similar to that previously described [1]. Briefly, female Wistar rats of 175±25 g were housed in standard conditions, 5 in each cage, and the treatments were allocated at random. The animals were given 100,000 IU (2.5 mg) of vitamin D_3 orally per rat and day, for 5 days. The phosphonates were administered daily by subcutaneous injections at 1 or 10 mg of phosphorus (P)/kg body weight (BW) starting 2 days before vitamin D_3 administration, and continuing for 8 days until the animals were killed. After sacrifice the aortas and

Kidney			
1 mg P/kg BW s.c.		10 mg P/kg BW s.c.	
0.91±0.33[1]	(6)	0.54±0.08[1]	(10)
2.18±0.49[2]	(9)	1.20±0.13[1]	(8)
1.28±0.41[1]	(8)	4.55±4.07[1]	(8)
5.68±1.72	(10)	2.33±0.67	(3)
5.03±2.21	(9)	2.83±0.88	(4)
0.44±0.04[1]	(7)		
1.33±0.56	(15)	significant difference $\alpha < 0.002$	
10.06±1.69	(41)		
0.81±0.27[1]	(10)	0.78±0.41[1]	(9)
1.89±0.54	(9)	1.55±0.41	(9)
4.58±1.93	(9)	2.15±0.97	(10)
2.43±1.04[2]	(10)	2.82±0.11	(10)
2.33±0.75	(10)	1.22±0.28[2]	(10)
1.46±0.69	(9)	significant difference $\alpha < 0.01$	
5.74±1.22	(34)		

kidneys were removed and their dry weights determined. The organs were then ashed and the residues dissolved in 0.5 *N* HCl. The calcium of the aortas, the kidneys and in plasma was determined by atomic absorption spectroscopy.

Two separate experiments were performed, five diphosphonates being compared in the first experiment and four in the second one. In each experiment a group which did not receive vitamin D_3, a group receiving vitamin D_3 alone, and a group with vitamin D_3 and the diphosphonate Cl_2MDP, a potent inhibitor of calcification in this system as previously described [1], were included.

The differences between the values obtained in the vitamin D_3 group and the group without vitamin D_3 as well as the groups receiving vitamin D_3 and the diphosphonates were examined statistically. As the values for aorta and kidney calcium followed a skewed distribution the nonparametric U test of Wilcoxon, Mann and Whitney was applied.

Effects on Rise in Plasma Calcium Induced by PTH in Thyroparathyroidectomized Rats

This method has also been described earlier [6]. In brief, male Wistar rats of 110±20 g were thyroparathyroidectomized (TPTX). From this moment they were changed from the diet containing 1.2% Ca and 1.1% P on which they were raised to a diet containing 0.18% Ca and 0.22% P. Furthermore, their drinking water was supplemented with calcium gluconate (2% w/v) starting immediately after TPTX until 6 days later. On the 4th day after TPTX the animals were caged in groups of 5 and the treatment with phosphonates was allocated at random and given daily over the next 3 days. From the 6th day after TPTX the animals continued to eat, but were allowed to drink distilled water only. On the 8th day after TPTX no phosphonates were given. A blood sample was taken in the morning and the animals were injected once subcutaneously with 50 USP parathyroid extract (PTH, Parathor-mone, Lilly, Indianapolis, USA) per rat. 6 h after the injection of PTH a second blood sample was taken. Plasma calcium was determined by atomic absorption spectroscopy and plasma phosphate by a modification [16] of the phosphomolybdate method of *Chen* et al. [17].

As mentioned above, two separate experiments were performed. Five diphosphonates were compared in the first and four in the second experiment. In each experiment a group receiving no PTH, a group receiving PTH alone, and a group receiving PTH and the diphosphonate Cl_2MDP, which we have previously shown to be active in this system [6], were included. The differences between the mean plasma calcium values in the animals receiving PTH alone and those receiving no PTH or PTH plus diphosphonates were examined statistically, using the two-sided Wilcoxon test again.

Chemicals

All reagents of analytical grade were purchased from Merck, Darmstadt, FRG, and vitamin D_3 was obtained from Wander, Berne, Switzerland. The diphosphonates were a gift from the Procter & Gamble Company, Cincinnati, USA. Their formulae are given

in table I. All polyphosphonates were dissolved in water or NaCl solution as appropriate to reach isotonicity and the pH was adjusted to 7.4. HDDP which was insoluble at pH 7.4 was left at pH 3.5. The concentrations of the compounds were such that the daily dose was given in 0.2 ml/100 g BW.

Results

Effect on Aortic Calcification

The results are shown in table II. The calcium content of the aortas in the groups without vitamin D_3 was not significantly different in the two experiments. In the second experiment aortic calcification in the group receiving vitamin D_3 alone was lower; this was, however, not statistically significant. The difference between the two groups with and without vitamin D_3 was highly significant in both experiments.

In the first experiment, Cl_2MDP significantly inhibited aortic calcification at both doses. In the second experiment, the inhibition was only significant with the lower dose of Cl_2MDP. At the higher dose no significance was obtained because of one extremely high value (69.4 µg Ca/mg aorta). Thus, high biological variation explains why some compounds are effective at low but not at high doses.

All compounds characterized by a P-C-P structure (geminal diphosphonates) and having free phosphate groups inhibited calcification. In contrast, condensate II, in which there are P-C-P groups, but in which two of the phosphates form a diphosphate, was not active. The vicinal polyphosphonates characterized by a P-C-C-P grouping (DCEDP and PTP) were not effective. Weak effects were observed with HFCPDP which has vicinal phosphates on a pentane ring, and TPMA where the phosphates are linked to $N(CH_2)_3$. Thus, the geminal phosphonates have the strongest effects, but only HDDP resembles Cl_2MDP in activity. It was, however, toxic.

Effect on Kidney Calcification

As seen in table II, the calcium content of the kidneys in the control groups (no vitamin D_3) was not significantly different in the two experiments. Kidney calcification, as well as aortic calcification, was somewhat lower in the second experiment, but again this difference was not significant. Significant inhibition of calcification was caused by Cl_2MDP at both doses in both experiments. All geminal diphosphonates except CHMDP showed activity, including condensate II, although only to a small extent. Again only HDDP matched Cl_2MDP in potency. The vicinal polyphosphonates DCEDP and PTP had no effect. Some activity was observed with TPMA and also with HFCPDP.

Oral Administration

The first five compounds were also tested by administering them orally. Since the results do not alter the conclusions drawn from the subcutaneous administration, they are not given in extenso. A significant effect on aortic calcification was obtained at 10 mg P/kg BW with Cl_2MDP, PAMDP, PPDP, CHMDP and HDDP and on kidney calcification with Cl_2MDP, PAMDP, PPDP and HDDP. At the dose of 1 mg P/kg BW, only Cl_2MDP and HDDP were effective on aorta calcification and only HDDP inhibited kidney calcification.

Effect on PTH-Induced Change in Plasma Calcium in TPTX Rats

Effect on Initial Plasma Calcium before Administration of PTH (table III). Calcemia of the untreated control and the PTH alone groups was not significantly different. The initial plasma Ca in the groups treated with

Table III. Effect of various polyphosphonates on the change in plasma calcium measured before and after injection of parathyroid hormone into thyroparathyroidectomized rats; plasma calcium is given as mg Ca/100 ml ± 1 SEM (number of determinations corresponds to survival)

Compound	Dose in mg P/kg BW s.c.	Survival	Initial plasma Ca	Change in plasma Ca 6 h after PTH injection	
Experiment 1					
Cl_2MDP	1	8/10	6.07±0.23	0.64±0.20[3]	
	10	8/10	5.61±0.72	0.00±0.20[1]	
PAMDP	1	10/10	6.59±0.25	2.27±0.26	
	10	10/10	10.0 ±0.23[1]	1.65±0.23	
PPDP	1	10/10	6.46±0.25	1.56±0.31	
	10	9/10	7.20±0.75	−0.02±0.23[1]	
DCEDP	1	9/10	6.97±0.26	2.64±0.20	
	10	8/10	9.24±0.32[1]	2.19±0.27	
CHMDP	1	10/10	6.93±0.33	2.18±0.36	
	10	10/10	9.49±0.25[3]	1.64±0.22	
HDDP	1	9/10	5.59±0.22[3]	0.19±0.22[1]	
	10	0/10			
Untreated		15/15	6.73±0.24	−0.71±0.15	} significant difference,
PTH alone		44/47	6.69±0.19	2.07±0.20	} α <0.002
Experiment 2					
Cl_2MDP	1	9/10	4.85±0.16[1]	0.88±0.21[1]	
	10	6/10	5.16±0.55	0.44±0.18[1]	
HFCPDP	1	10/10	5.52±0.14	2.40±0.20	
	10	9/10	5.63±0.25	2.27±0.37	
PTP	1	8/10	5.90±0.26	2.20±0.49	
	10	10/10	5.89±0.21	2.82±0.31	
Condensate II	1	10/10	5.42±0.31	2.10±0.25	
	10	10/10	6.05±0.20	2.63±0.37	
TPMA	1	9/10	6.25±0.25	2.58±0.26	
	10	10/10	6.62±0.48	2.19±0.19	
Untreated		10/10	5.66±0.14	−0.58±0.16	} significant difference,
PTH alone		35/36	5.82±0.15	2.30±0.22	} α <0.002

[1] The difference between treatment mean and mean of PTH alone is significant α <0.002.
[2] α <0.01.
[3] α <0.05.

Cl_2MDP and HDDP was slightly decreased whereas that of the groups receiving PAMDP, DCEDP and CHMDP was increased, sometimes up to values similar to those seen in non-TPTX animals.

Effect on Change in Plasma Calcium 6 h after PTH Injection. PTH induced a significant increase in plasma Ca in both experiments. This increase was inhibited only by HDDP, Cl_2MDP and PPDP.

Discussion

These results confirm the earlier observations [1] that the geminal diphosphonates are the best inhibitors of calcification. They also confirm that the effect is dependent on the structure of the geminal diphosphonate. It is interesting that only long-chain diphosphonates matched Cl_2MDP in activity. Some of the compounds such as CHMDP and PAMDP, which were active in this study, also have been found to be active on bone and cartilage mineralization [18]. DCEDP, which was inactive, did, however, inhibit both cartilage and osteoid mineralization in a previous study [18]. This confirms previous findings that the inhibition of soft tissue calcification by a diphosphonate does not necessarily parallel the inhibition of hard tissue calcification. Thus, Cl_2MDP has been found to be an excellent inhibitor of calcification in soft tissues, but weak in bone and cartilage [2].

Only HDDP and PPDP inhibited the hypercalcemia induced by PTH besides Cl_2MDP, and only HDDP was more effective than Cl_2MDP. In this animal model, where the animals are fed a low Ca diet when PTH is administered, the hypercalcemia is likely to reflect a change in bone resorption [19]. This is supported by earlier findings that compounds active in this model system also were found to inhibit actual bone resorption as measured in various systems [2, 7, 20]. These compounds also reduce the initial calcemia, possibly by the same mechanism. In contrast, some other compounds increase the initial calcemia. Since these compounds are those which previously have been found to block bone and cartilage mineralization [18], the two effects might be related.

It is interesting that all these new effective compounds are geminal diphosphonates with an elongated side chain. These results open new possibilities into the search for future, more powerful, inhibitors of calcification and bone resorption.

Acknowledgements

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Roman C. Mühlbauer,
Department of Pathophysiology,
University of Berne,
Murtenstrasse 35,
CH-3010 Berne (Switzerland)

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Relation between bone mineralization, Ca absorption, and plasma Ca in phosphonate-treated rats

U. TRECHSEL, R. SCHENK, J.-P. BONJOUR, R. G. G. RUSSELL, AND H. FLEISCH
Department of Pathophysiology and Department of Anatomy, University of Berne, Berne, Switzerland

TRECHSEL, U., R. SCHENK, J.-P. BONJOUR, R. G. G. RUSSELL, AND H. FLEISCH. *Relation between bone mineralization, Ca absorption, and plasma Ca in phosphonate-treated rats.* Am. J. Physiol. 232(3): E298–E305, 1977 or Am. J. Physiol.: Endocrinol. Metab. Gastrointest. Physiol. 1(3): E298–E305, 1977. – Disodium ethane-1-hydroxy-1,1-diphosphonate (EHDP) is known to inhibit the crystallization of calcium phosphate salts in vitro. Large doses of EHDP administered in vivo inhibit skeletal mineralization, decrease intestinal calcium absorption, and produce hypercalcemia. In the present study, EHDP or one of 13 other phosphonates were given to rats at 10 mg P/kg·day sc for 7 days in order to better define the nature of the relationship between bone mineralization, intestinal absorption, and plasma calcium in the regulation of calcium homeostasis. Each of the phosphonates which inhibited skeletal mineralization in vivo also inhibited crystallization in vitro, but the converse was not true. A very close correlation was found between inhibition of skeletal mineralization, decreased intestinal calcium absorption, and slight hypercalcemia. A dose-response study with two compounds also revealed the same close correlation. It is argued that the impairment of intestinal calcium absorption in phosphonate-treated rats may represent a secondary homeostatic response to the primary effect of the drugs on bone mineralization. This response may be mediated by an elevation of a fraction of plasma calcium.

calcium homeostasis; plasma P_i; diphosphonates; cartilage mineralization; osteoid mineralization; bone resorption

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DISODIUM ETHANE-1-HYDROXY-1,1-DIPHOSPHONATE (EHDP) and certain other diphosphonates that contain P—C—P bonds are known to be potent inhibitors of the formation and dissolution of hydroxyapatite crystals in vitro (7–10, 18) and to inhibit bone turnover in vivo (11). In earlier studies a reasonable correlation could be found between the ability of particular diphosphonates to inhibit soft tissue calcification in vivo and their effect on apatite crystal growth in vitro (8).

EHDP in large doses (5–10 mg P/kg·day sc), in addition to its effects on soft tissue calcification, inhibits the mineralization of bone and cartilage and induces a form of rickets and a slight hypercalcemia in experimental animals (11, 15, 19). Also it has been found that EHDP in doses that inhibit bone calcification caused an impairment of the intestinal capacity for calcium absorption (3, 11, 16). This effect on intestinal calcium absorption appears to be due to diminished renal production of 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), one of the major biologically active metabolites of vitamin D_3. Treatment with rachitogenic doses of EHDP impairs production of 1,25-$(OH)_2D_3$ in both rats and chicks (1, 14), and the intestinal capacity for calcium absorption can be restored to normal in EHDP-treated rats by giving small doses of 1,25-$(OH)_2D_3$ (2).

The mechanism by which this inhibition of 1,25-$(OH)_2D_3$ production is induced in EHDP-treated animals is unknown. There may be a direct effect of EHDP on the synthesis of 1,25-$(OH)_2D_3$ in the kidney, but for several reasons it seems more likely that the reduction in 1,25-$(OH)_2D_3$ represents a secondary homeostatic response which serves to protect the organism from being overloaded with calcium after the drug has inhibited calcium entry into bone. One change that could mediate this response is the rise in plasma calcium seen in EHDP-treated rats.

In the present paper we have attempted to test this hypothesis by studying the relationships between the effects of a series of phosphonates of different structure on the formation of calcium apatite crystals in vitro and on bone mineralization, intestinal calcium absorption, and plasma calcium in vivo. Indeed, if a constant relationship between these four variables is found with a considerable number of compounds, this would be highly suggestive of a homeostatic link between them. Such a homeostatic mechanism could operate physiologically.

Nine of the compounds studied, including EHDP, contained a P—C—P bond which is thought to be essential for the inhibition of crystal formation (8, 10); the other five phosphonic acids chosen had a variety of other structures. It was found that there was a good correlation between the inhibition of crystal growth in vitro and the inhibition of bone mineralization, the decrease in intestinal absorption of calcium, and the rise in blood calcium in vivo. These correlations imply that there is a functional relationship between these parameters and support the hypothesis that the primary effect of the diphosphonates on bone is due to their physicochemical action to inhibit calcification. The decrease in intestinal calcium absorption appears to be a homeostatic response, possibly mediated by hypercalcemia.

MATERIALS

Table 1 shows the formulas of the phosphonic acids used in this study. The compounds were generous gifts

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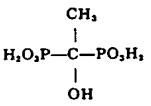
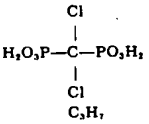
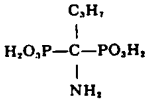
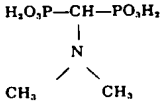
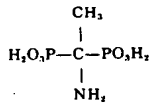
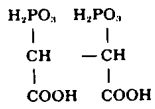
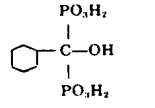
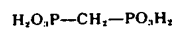
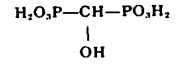
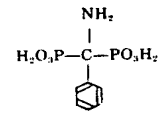
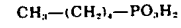
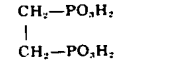
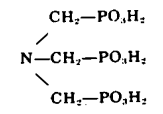
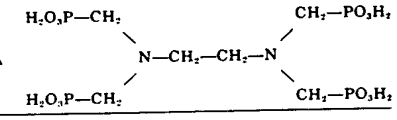
TABLE 1. *List of phosphonates studied*

Ethane-1-hydroxy-1,1-diphosphonic acid	EHDP	
Dichloromethane diphosphonic acid	Cl_2MDP	
1-Aminobutane,1,1-diphosphonic acid	AB-1,1-DP	
N,N-dimethylamino-methane diphosphonic acid	DMAMDP	
1-Aminoethane-1,1-diphosphonic acid	AEDP	
1,2-Dicarboxyethane-1,2-diphosphonic acid	DCEDP	
Cyclohexylhydroxy-methane diphosphonic acid	CHMDP	
Methane diphosphonic acid	MDP	
Hydroxymethane diphosphonic acid	HMDP	
Phenylaminomethane diphosphonic acid	PAMDP	
n-Pentane monophosphonic acid	PMP	
Ethane-1,2-diphosphonic acid	E-1,2-DP	
N,N,N-triphosphono-methylamine	TPMA	
N,N-tetraphosphono-methylethylene-diamine	TePMEDA	

from The Procter & Gamble Company, Cincinnati (EHDP, Cl_2MDP, AEDP, DCEDP, CHMDP, MDP, HMDP, PAMDP, PMP, E-1,2-DP), and from Henkel & Cie GMBH, Dusseldorf, Germany (AB-1,1-DP, DMAMDP, TPMA, TePMEDA).

METHODS

Effects of Phosphonates on Formation of Apatite Crystals in vitro

The minimum product (formation product) [Ca] × [P], required to induce precipitation of calcium phosphate from solution, was measured by a slight modification of a method described previously (6). Ca, 1.7 mM, was incubated with increasing amounts (0.5–8.0 mM) of phosphate in plastic vessels on a shaker for 3 days at 37°C. The solutions were buffered at pH 7.4 with 0.01 M barbital, and the ionic strength was adjusted to 0.16 by adding the appropriate amount of KCl. After 3 days any crystals formed were removed by centrifugation, and the amounts of calcium remaining in solution were compared with those present in vials incubated without the agent, thus permitting the determination of the minimum [Ca] × [P] product required to induce precipitation.

Animal Experiments

Male or female Wistar rats bred in this laboratory were raised and maintained on a commercial diet (Altromin no. 1314), containing 1.1% Ca, 1.2% P, and 2,800 IU vitamin D_3/kg. Rats weighing 160–180 g were used for 19 separate experiments. In a first series of 12 experiments, the compounds were tested at a fixed dose level of 10 mg P/kg body wt sc, one or two substances being studied per experiment. In 11 of the experiments, EHDP was also tested, so that the reproducibility of the results was assessed not only in control (NaCl-treated) animals, but also in animals treated with a highly active compound. In a second series of seven experiments, the effects of different doses of EHDP and of DMAMDP were tested.

In each experiment the rats received subcutaneous injections of a particular phosphonate once daily (A.M.) for 7 days. For the dose of 10 mg P/kg, the phosphonic acids were dissolved in distilled water, and the solution was adjusted to pH 7.4 with 1 N NaOH and injected in a volume of 2 ml/kg body wt. When lower doses were used, the volume injected was kept constant by diluting with 0.15 M NaCl. Control rats were injected with an equivalent volume of 0.15 M NaCl. When doses above 10 mg P/kg were given, the volume injected was increased.

After the seventh injection, the animals were fasted overnight. On the next day the intestinal absorption capacity for calcium was measured as described below. Then the animals were killed, and both tibias were removed for measurement of the width of the epiphyseal plates and for histological examination.

Blood samples for the determination of calcium and inorganic phosphate were taken from the tip of the tail on the 3rd day of treatment in a nonfasted state before and 1 h after the injection of the drug, and on the last, i.e., 8th day, after the animals had been fasted overnight just before the measurement of the intestinal calcium absorption capacity. These times were chosen on the basis of previous experiments with EHDP, in which two types of hypercalcemia could be distinguished: an acute rise in plasma calcium shortly after

the injection of the drug and a chronic elevation which appeared after several days of treatment. In the present series of experiments, we measured the acute hypercalcemic response by the change of plasma calcium 1 h after the third injection of the drug, and we measured the chronic response by the preinjection value for plasma calcium on the 3rd day and the value in the fasted animal before the tied-loop assay.

Measurement of Intestinal Absorption Capacity for Calcium

The animals were anesthetized with an intraperitoneal injection of 35–40 mg/kg of pentobarbital (Nembutal, Abbott Laboratories), and the calcium absorption capacity was estimated by a modification of the tied-loop in situ technique (17, 25) described in detail previously (2). The first 8–12 cm of the small intestine, referred to as the duodenum, were rinsed with cold saline, and the segment was closed at the distal end. Then it was filled with 0.5 ml of a solution containing 150 mM NaCl, 0.4 mM $CaCl_2$, and 0.1 μCi/0.5 ml ^{45}Ca (sp act 30 mCi/mg), and the segment was closed at the proximal end just beyond the entry of the bile duct. After 15 min the loop was excised and the contents emptied into a test tube. The ^{45}Ca activity in the luminal fluid was determined, and the fraction of ^{45}Ca that had disappeared from the lumen during the incubation could then be calculated. The intestinal segments were blotted and then ashed in order to measure the ^{45}Ca content in the tissue. The percentage of the initial amount of ^{45}Ca found in the intestinal wall, namely 8.25 ± 1.91% (mean ± SD), was not altered by treatment with phosphonates. Therefore, the differences in the percentage of ^{45}Ca which disappeared from the tied loops during the incubation is a good measure of the difference in the percentage of tracer transported beyond the intestinal wall.

Measurement of Width of Epiphyseal Plate

Three frontal sections of the upper part of one tibia of each rat were stained according to Von Kossa's technique. The width of the epiphyseal plate was measured under the microscope in three different parts of every section, and the mean of the measurements was taken.

Histological techniques used for examination of the proximal tibia by microradiography and by light microscopy were essentially the same as those described in detail elsewhere (19).

Analytical Methods

Plasma calcium was determined by atomic absorption (Perkin Elmer Corp. model 290 B) after dilution of the sample with 0.5% $LaCl_2$. Inorganic phosphate was measured using the method of Chen et al. (5). The ^{45}Ca activities were measured on a Packard Tri-Carb liquid scintillation counter (model 2425).

Statistical Analysis

Effects of treatment with various phosphonates at a dose of 10 mg P/kg body wt. The experimental results were expressed as mean ± standard error, and the significance of the differences between groups was determined by the Student *t* test for unpaired values. The values for plasma calcium and inorganic phosphate of treated animals were compared with the pooled control values of all experiments. This was also done for calcium absorption, inasmuch as the mean control values in different experiments were 84.53 ± 3.05% (mean ± SD) and therefore very reproducible. However, the mean control value for the width of the epiphyseal plates was less constant, and therefore the values in the treated animals were compared with the control values from the same experiment.

Effect of different doses of DMAMDP and EHDP. These experiments were analyzed separately. Results of the different experiments in the dose-response study were pooled and analyzed as above. The calcium absorption results were expressed as a ratio to control, taking the mean control value for each experiment as 1.0.

RESULTS

Effects of Phosphonates on Formation Product of Apatite Crystals in vitro

The results are shown in Table 2. PMP and E-1,2-DP were the only compounds that had minimal effects at 10^{-6} and 10^{-5} M. Cl_2MDP and DMAMDP were highly active at 10^{-5} M, whereas all the other compounds produced a marked increase of the formation product at 10^{-6} M. EHDP and TePMEDA were still very active at 10^{-7} M.

Effects of Phosphonate Treatment at a Dose of 10 mg P/kg Body Wt

The effect of treatment with phosphonates on the mineralization of the epiphyseal plate of the tibia is shown in Table 3 and Fig. 1. Nine of the compounds,

TABLE 2. *Influence of various phosphonates on minimum product [Ca] × [P] (formation product) necessary to induce precipitation of calcium phosphate from solution*

Compound	Mean Increase in Formation Product over Control Value		
	10^{-5} M*	10^{-6} M*	10^{-7} M*
		mM^2	
EHDP		>7.30 (6)†	1.88 (5)†
Cl_2MDP	5.30 (1)†	+1.10 (1)†	
AB-1,1-DP		>3.83 (2)	1.21 (1)
DMAMDP	>3.63 (1)	+1.01 (2)	
AEDP		+4.90 (1)†	0.30 (1)†
DCEDP		+3.54 (1)	
CHMDP		+3.58 (1)	
MDP		+2.21 (7)†	0.62 (5)†
HMDP		+3.10 (1)†	0.40 (1)†
PAMDP	>4.03 (1)	>4.03 (1)	1.21 (1)
PMP	+0.38 (6)†	+0.47 (8)†	
E-1,2-DP	+0.50 (1)†	+0.20 (1)†	
TPMA	>3.63 (1)	+1.81 (2)	
TePMEDA		>3.43 (2)	2.02 (1)

Numbers of experiments are shown in parentheses. *Concentration of phosphonate. †Results published by Fleisch et al. (8).

TABLE 3. *Effect of 7 days of phosphonate treatment on bone mineralization and bone resorption and on disappearance of ^{45}Ca from isolated duodenal loops in situ*

Treatment	No. of Animals	Width of Epiphyseal Plate, μm	Histology			No. of Animals	^{45}Ca Disappeared from Ligated Segment, % Initial Activity
			Inhibition of mineralization in tibial epiphysis and metaphysis		Inhibition of resorption in metaphysis		
			Cartilage	Osteoid			
EHDP	38	1,374±32[a]	+	+	+	37	50.4±1.4[a]
Cl_2MDP	4	462±37	−	−	+	4	91.4±1.7[c]
AB-1,1-DP	4	1,025±103[b]	+	+	−	4	58.1±10.3[a]
DMAMDP	12	810±58[a]	(+)[c]	+	−	12	81.5±1.5
AEDP	4	1,162±97[a]	+	+	−	4	40.7±8.0[a]
DCEDP	4	775±97[b]	(+)[c]	+	−	3	45.3±4.3[a]
CHMDP	4	1,487±31[a]	+	+	−	4	53.1±4.9[a]
MDP	8	362±18	−	+	+	7	69.3±2.3[a]
HMDP	3	733±44[b]	(+)[c]	+	+	2	51.1±7.6[a]
PAMDP	4	1,200±79[a]	+	+	+	4	53.9±5.9[a]
PMP	8	451±30	−	−	−	8	82.2±2.5
E-1,2-DP	4	475±25	−	−	−	4	91.7±2.4[c]
TPMA	4	544±67	−	(+)[d]	+	4	81.0±3.9
TePMEDA	4	1,275±105[a]	+	+	+	4	55.5±1.1[a]
Control	47	411±14	−	−	−	42	84.5±1.0

Values are means ± SE. Phosphonate treatment, 10 mg P/kg·day sc. [a] $P < 0.001$. [b] $P < 0.01$. [c] Locally inhibited. [d] In two animals, toward the end of the experimental period. [c] $P < 0.05$.

EHDP, AB-1,1-DP, AEDP, DMAMDP, DCEDP, CHMDP, HMDP, PAMDP, and TePMEDA, produced a significant enlargement of the epiphyseal plate after 7 days of treatment. MDP did not produce an enlargement of the epiphyseal plate as measured by the macroscopic Von Kossa technique, but detailed histology showed that the mineralization of newly formed osteoid was inhibited, even though the mineralization of the cartilage in the hypertrophic zone of the epiphyseal growth plate was normal. The histological examination of the bones also revealed that the enlargement of the epiphyseal plate after treatment with DMAMDP, as measured by our technique, overestimated the effect of this drug, because the enlargement was visible only at the periphery of the plate, the center appearing normal. For all the other compounds, the Von Kossa stain was a reliable index of the mineralization of the epiphyseal plate.

Seven of the compounds significantly inhibited remodelling of the metaphysis by inhibiting the resorption of cartilage and bone. In one case (Cl_2MDP) this took place without accompanying inhibition of mineralization, whereas with the others (EHDP, MDP, HMDP, PAMDP, TPMA, and TePMEDA) inhibition of osteoid or of osteoid and cartilage mineralization occurred.

Table 3 also shows the results for intestinal absorption of calcium. EHDP, AB-1,1-DP, AEDP, DCEDP, CHMDP, HMDP, PAMDP, and TePMEDA all produced a similar, large decrease of the calcium absorption capacity of the duodenum. MDP produced a significant but weaker effect. Cl_2MDP and E-1,2-DP produced a small, but statistically significant increase of the calcium absorption, whereas the remaining compounds had no effect.

The values for plasma Ca are presented in Table 4. Three values are shown: *1*) the plasma Ca before the third injection of the drug, *2*) the change 1 h after the third injection, and *3*) the value in the fasted animals 24 h after the seventh injection and just before the tied-loop assay. EHDP, AB-1,1-DP, AEDP, DCEDP, CHMDP, HMDP, PAMDP, PMP, and TePMEDA produced an elevation of one, two, or all three values. In contrast AEDP, HMDP, and PMP caused a significant decrease in one or more of the values, but the remaining compounds did not change plasma calcium.

Table 5 shows the values for inorganic phosphate corresponding to the calcium values shown in Table 4. Among the preinjection values on the 3rd day of treatment, the majority were decreased. One hour after the injection, TPMA produced a significant increase and HMDP produced a decrease in plasma phosphate. In the fasted animals at the end of the experiment, three of the phosphonates produced values significantly lower, and three significantly higher than the control values.

Effects of Different Doses of DMAMDP and EHDP

Figure 2 shows the effects of 1, 10, and 30 mg P/kg·day DMAMDP. P, 10 mg/kg·day, produced an enlargement of the epiphyseal plate, but, as mentioned above, this value overestimates the effect of the drug on bone mineralization at this dose level. With the higher dose of 30 mg P/kg, there was a clear effect on all three variables.

A similar study using EHDP is shown in Fig. 3. The effects on skeletal mineralization, intestinal absorption, and plasma calcium all began to appear at the same dose level, namely 5 mg P/kg·day. However, with a dose of 5 mg P/kg, the preinjection plasma calcium was not significantly increased, whereas at 10 mg P/kg all three values of plasma calcium were elevated.

Body Weight Gain and Toxicity

Although the animals were not pair fed, body weight was recorded during treatment. The following compounds significantly inhibited weight gain: EHDP, AB-1,1-DP, AEDP, DCEDP, CHMDP, HMDP, and TePMEDA. E-1,2-DP caused a significant increase in body weight. Histologically there was no evidence for a decreased growth of the tibia, and the effects on mineralization cannot be attributed to effects on growth.

DISCUSSION

Several findings are of interest concerning the effect of the phosphonates on skeletal mineralization. There is a good correlation between their effect on crystal formation in vitro and the inhibition of epiphyseal mineralization in the sense that all the compounds that inhibited bone mineralization also inhibited crystal formation in vitro. The reverse, however, was not true, since Cl_2MDP was an active inhibitor of crystal formation in vitro, but did not inhibit the bone mineralization process in vivo.

Five of the compounds tested did not contain the P—C—P bond of the geminal diphosphonates. DCEDP and E-1,2-DP are vicinal diphosphonates, the two phosphate

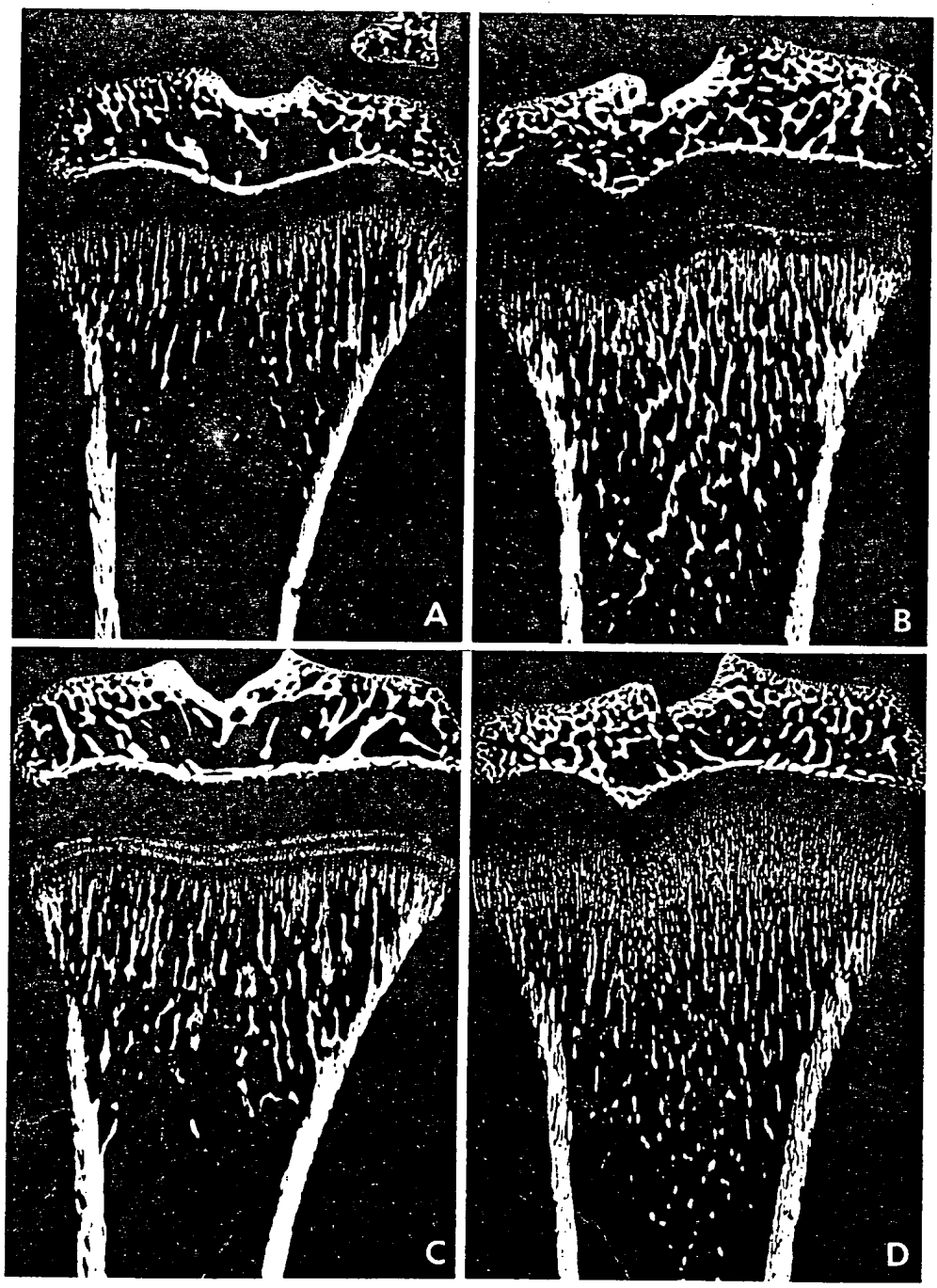


FIG. 1. Microradiographs of proximal ends of rat tibias. *A*: control. *B*: treatment with 10 mg P/kg·day sc of MDP. Inhibition of bone and calcified cartilage resorption, inhibition of bone mineralization, but undisturbed cartilage mineralization. This results in a cylindrical shape of metaphyseal segment formed under treatment. Densely packed, thin mineralized septa correspond to calcified cartilage coated with some osteoid. No extention of marrow cavity in proximal direction. *C*: treatment with 10 mg P/kg·day sc of EHDP. Inhibition of bone and cartilage resorption, combined with an inhibition of both bone and cartilage mineralization. Transverse low-density lines in metaphysis indicate a temporary arrest of bone mineralization immediately after injections on 1st and 2nd day. The third and following injections led to a complete arrest of mineralization and consecutive widening of the growth plate. *D*: treatment with 10 mg P/kg·day sc of Cl_2MDP. Inhibition of bone and calcified cartilage resorption, undisturbed bone and cartilage mineralization. Cylindrical shape of metaphysis, increased number of trabecular, normal width of growth plate.

groups being attached on two neighboring carbons (P—C—C—P). Interestingly DCEDP, in which there are two carboxyl groups as well as two phosphoric acid groups, is an active inhibitor of crystal formation in vitro and of skeletal mineralization in vivo, whereas E-1,2-DP had no effect. Both TPMA and TePMEDA which do not contain the P—C—P structure of the active diphosphonates were inhibitors of crystal formation in vitro, and TePMEDA was also particularly active in vivo. These results support the hypothesis (8, 10) that the inhibition of crystal formation and of bone mineralization depends on the presence of at least two $—PO_3H_2$ groups in a well-defined steric relationship similar to that present in the P—O—P bond of pyrophosphate.

There was a clear association between the effects on bone and gut because all the treatments that inhibited the mineralization of osteoid or of osteoid and cartilage also produced an impairment of the intestinal capacity for calcium absorption. In general, the mineralization of osteoid was more sensitive to inhibition by phosphonates than was the mineralization of cartilage, and those doses of phosphonates (e.g., MDP at 10 mg P/kg) that inhibited osteoid alone rather than both osteoid and cartilage were associated with a smaller effect on intes-

TABLE 4. *Plasma calcium at three different times during phosphonate treatment*

Treatment	Plasma Calcium Before Third Injection		Change in Plasma Calcium 1 h After Third Injection		Plasma Calcium 24 h After 7th Injection	
	No. of animals	mg/100 ml	No. of animals	mg/100 ml	No. of animals	mg/100 ml
EHDP	39	10.88 ±0.10[b]	39	+2.10 ±0.13[b]	34	10.66 ±0.07[b]
Cl_2MDP	4	10.09 ±0.18 NS[a]	4	−0.15 ±0.20 NS	4	10.22 ±0.25 NS
AB-1,1-DP	4	11.21 ±0.08[b]	4	+0.06 ±0.25 NS	4	10.74 ±0.29[c]
DMAMDP	12	10.17 ±0.14 NS	12	+0.11 ±0.20 NS	12	9.92 ±0.10 NS
AEDP	4	10.43 ±0.11 NS	4	−0.40 ±0.05[d]	4	11.09 ±0.21[b]
DCEDP	4	11.13 ±0.30[c]	4	−0.39 ±0.43 NS	4	10.15 ±0.36 NS
CHMDP	4	11.74 ±0.18[b]	4	+0.55 ±0.17[c]	4	10.74 ±0.16[c]
MDP	8	10.42 ±0.11 NS	8	+0.04 ±0.12 NS	4	9.99 ±0.04 NS
HMDP	4	9.40 ±0.34[b]	3	+1.86 ±0.59[b]	2	9.43 ±0.01[c]
PAMDP	4	10.66 ±0.13 NS	4	+0.19 ±0.12 NS	4	10.98 ±0.25[b]
PMP	8	10.72 ±0.08[d]	8	−0.48 ±0.27[c]	4	10.28 ±0.14 NS
E-1,2-DP	4	10.10 ±0.17 NS	4	+0.08 ±0.24 NS	4	10.26 ±0.32 NS
TPMA	4	10.43 ±0.15 NS	4	+0.23 ±0.13 NS	4	9.79 ±0.12 NS
TePMEDA	4	11.34 ±0.20[b]	4	+0.38 ±0.17 NS	4	10.73 ±0.09[c]
Control	47	10.29 ±0.07	47	+0.01 ±0.06	42	10.16 ±0.06

Values are means ± SE. Phosphonate treatment, 10 mg P/kg · day sc. Level of significance of the difference versus pooled control values of all experiments by the Student *t* test for unpaired values. [a]NS, $P > 0.05$. [b]$P < 0.001$. [c]$P < 0.01$. [d]$P < 0.05$.

tinal calcium absorption capacity. The association between the effect on skeletal mineralization and on calcium absorption was also demonstrated in the dose-response study using DMAMDP and EHDP, although DMAMDP in a dose of 10 mg P/kg tended to produce an enlargement of the epiphyseal plate without affecting calcium absorption. In a preliminary report (23), we interpreted this finding with DMAMDP as an indication that the two effects could be dissociated. However, subsequent detailed histological examination revealed that we had overestimated the effect on epiphyseal mineralization so that there is no clear evidence for such a dissociation.

It is interesting to speculate about the mechanism by which the effect of phosphonate treatment on bone mineralization and on intestinal calcium absorption might be linked. The impairment of calcium absorption in EHDP-treated animals is the result of a decreased production of 1,25-$(OH)_2D_3$ in the kidney (1, 2, 14). Although 1,25-$(OH)_2D_3$ was not measured directly in the present study, it is reasonable to suggest that the other phosphonates impair the capacity for intestinal calcium absorption by the same mechanism.

The mechanism by which the renal 1-hydroxylase might be inhibited is obscure. A direct effect of EHDP on the enzyme, which has been demonstrated in vitro using high concentrations of EHDP (1, 13), seems an unlikely explanation for several reasons. The inhibitory effect of the drug given in vivo has been shown to depend on the animal's vitamin D status (14), as well as on dietary calcium (22), and is therefore more likely to be indirect. Furthermore, the striking correlation between the effects on skeletal mineralization, on intestinal absorption, and on plasma calcium suggests that the three events are associated. Rather than propose that each phosphonate has independent effects on each of these changes, a simple explanation is that there is a single primary effect on one of these events which leads to secondary changes in the others. Previous studies with EHDP suggest that inhibition of skeletal mineralization occurs very rapidly and may be the earlier change, whereas the effect on intestinal calcium absorption takes 3 days to reach its maximum. Since plasma calcium has been proposed to play an important role in the regulation of 1,25-$(OH)_2D_3$ production under physiological conditions (4), the slight hypercalcemia that is present whenever intestinal absorption is decreased could be the signal that mediates the change in the intestine when skeletal mineralization is inhibited. The rise in plasma calcium may be related directly to im-

TABLE 5. *Plasma inorganic phosphate at three different times during phosphonate treatment*

Treatment	Plasma Inorganic Phosphate Before Third Injection		Change in Plasma Inorganic Phosphate 1 h After Third Injection		Plasma Inorganic Phosphate 24 h After Seventh Injection	
	No. of animals	mg/100 ml	No. of animals	mg/100 ml	No. of animals	mg/100 ml
EHDP	37	9.98 ±0.13 NS[a]	36	−0.33 ±0.15 NS	34	8.96 ±0.22[b]
Cl_2MDP	4	8.41 ±0.12[b]	4	−0.43 ±0.31 NS	4	8.43 ±0.22[d]
AB-1,1-DP	4	8.45 ±0.42[b]	4	−0.46 ±0.16 NS	4	9.74 ±0.42 NS
DMAMDP	11	8.48 ±0.19[c]	11	+0.03 ±0.20 NS	12	9.57 ±0.23 NS
AEDP	4	7.66 ±0.13[c]	4	+0.39 ±0.36 NS	4	11.01 ±0.71[d]
DCEDP	4	8.41 ±0.22[b]	4	−0.18 ±0.30 NS	4	10.75 ±0.16 NS
CHMDP	4	9.37 ±0.47 NS	4	−0.11 ±0.45 NS	3	9.31 ±0.91 NS
MDP	8	8.49 ±0.22[c]	8	−0.27 ±0.19 NS	4	9.21 ±0.08 NS
HMDP	3	8.70 ±0.32[d]	2	−2.07 ±0.66[b]	2	7.18 ±0.18[c]
PAMDP	4	10.17 ±0.38 NS	4	−0.31 ±0.40 NS	4	9.09 ±0.81 NS
PMP	8	9.74 ±0.34 NS	8	−0.18 ±0.30 NS	4	11.06 ±0.03[d]
E-1,2-DP	4	10.10 ±0.45 NS	4	−0.33 ±0.42 NS	4	9.88 ±0.78 NS
TPMA	3	8.57 ±0.26[d]	3	+0.88 ±0.20[b]	3	9.95 ±0.26 NS
TePMEDA	4	10.65 ±0.23 NS	4	−0.61 ±0.18 NS	4	11.07 ±0.31[d]
Control	47	9.76 ±0.13	47	−0.37 ±0.11	41	9.84 ±0.16

Values are means ± SE. Phosphonate treatment, 10 mg P/kg · day sc. Level of significance of the difference versus pooled control values of all experiments by the Student *t* test for unpaired values. [a]NS, $P > 0.05$. [b]$P < 0.01$. [c]$P < 0.001$. [d]$P < 0.05$.

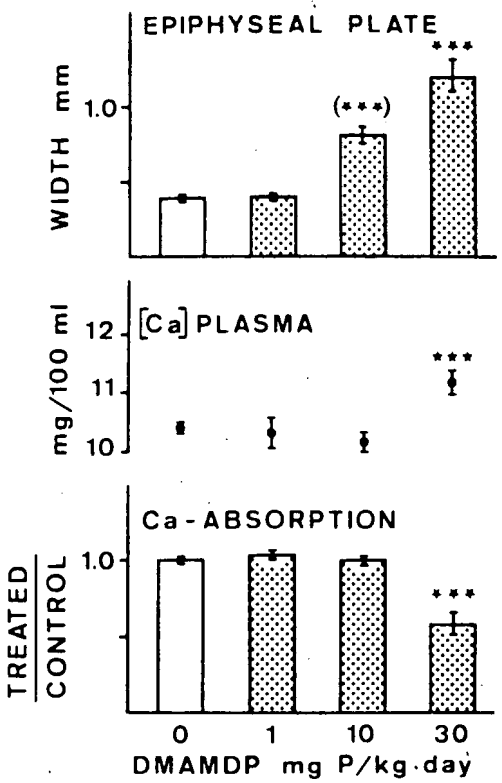


FIG. 2. Effects of DMAMDP given at a dose of 1 (n = 8), 10 (n = 12), and 30 (n = 8) mg P/kg·day for 7 days. Values are means ± SE. ***, $P < 0.001$ as compared to controls (n = 23). Results of 6 experiments were pooled. Tied-loop results were expressed in a standard way by dividing all values of an experiment by mean value of controls of same experiment. Values of plasma calcium are preinjection values on 3rd day of treatment.

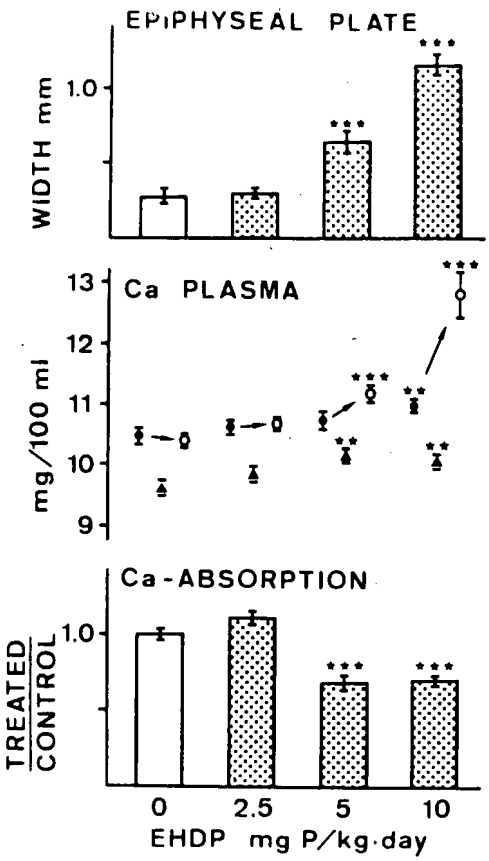


FIG. 3. Effects of EHDP given in a dose of 2.5, 5, and 10 mg P/kg·day sc for 7 days. There were 12 animals in each group. Values are means ± SE. **, $P < 0.01$, ***, $P < 0.001$ as compared to controls. Results of 4 separate experiments were pooled. Tied-loop results were expressed in a standard way by dividing all values of an experiment by mean value of controls of same experiment. Three values of plasma calcium are indicated, namely preinjection (closed circles) and 1-h postinjection (open circles) values on 3rd day of treatment, and value in fasted animals (triangles) before tied-loop assay.

paired entry of calcium into the skeleton. Of interest, the acute increase of plasma calcium has been found to be due solely to an increase of a nonionized, nonultrafiltrable component. Similar results were obtained by Gitelman (12) in dogs infused with large amounts of EHDP. It is thus tempting to speculate that the calcium complex found in plasma from EHDP-treated rats might be directly involved in the link between skeletal mineralization and calcium absorption.

No correlation was found between plasma inorganic phosphate and the effects of phosphonate treatment. This is of interest, because plasma inorganic phosphate has also been suggested to play a role in the regulation of 1,25-$(OH)_2D_3$ production (21). Although our results suggest that the plasma concentration of P_i is unlikely to play a role in the impairment of calcium absorption in the intestine of phosphonate-treated rats, it is possible that changes in intracellular phosphate might be involved. There is evidence that the distribution of inorganic phosphate is altered during treatment with phosphonates in experimental animals and humans (20, 24).

In conclusion, we have studied the influence of 14 phosphonates on bone mineralization, intestinal calcium absorption, and plasma Ca. The effects of each compound on the three variables were closely interrelated, suggesting a homeostatic link between them. The inhibition of bone mineralization is likely to be the primary effect of the drugs, inasmuch as this correlates well with their physicochemical action on hydroxyapatite crystal formation. The impairment of intestinal calcium absorption could then be considered to be a secondary homeostatic response to the decrease of calcium deposition into bone, protecting the organism from being overloaded with calcium. The factor that might mediate this response could be an increase in some fraction of plasma calcium.

These studies may indicate a new way of approaching the relationship between skeletal mineralization and intestinal absorption of calcium and provide a useful basis for the choice of certain phosphonates as potential therapeutic agents against disorders of calcium metabolism in humans.

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The Inhibitory Effect of Phosphonates on the Formation of Calcium Phosphate Crystals *in vitro* and on Aortic and Kidney Calcification *in vivo*

H. A. Fleisch, R. G. G. Russell, S. Bisaz, R. C. Mühlbauer
Department of Pathophysiology, University of Berne, Berne, and Laboratory for Experimental Surgery, Davos, Switzerland
D. A. Williams
Department of Statistics, University of Edinburgh, Edinburgh, Scotland

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Abstract. 1. Various phosphonates, which are compounds containing C-P bonds, have been studied to see whether they are able to inhibit, in a manner similar to that of pyrophosphate and the condensed phosphates, the crystallization of calcium phosphate *in vitro* and the pathological calcification of the aorta and the kidneys of rats given large amounts of vitamin D_3.

2. Six of the ten compounds studied markedly increased the minimum product, [Ca] × [P], required to induce the precipitation of calcium phosphate *in vitro* under physiological conditions of pH, ionic strength and temperature. Inhibition was observed at concentrations as low as 10^{-7}—10^{-6} M.

3. Most of the diphosphonates, particularly those possessing P-C-P bonds, showed some ability to inhibit the calcification of the aortas and kidneys of rats treated with large amounts of vitamin D_3. The most effective inhibitors were methylene diphosphonate (MDP), ethane-1-hydroxy-1:1-diphosphonate (EHDP) and dichloromethylene diphosphonate (Cl_2MDP).

4. The phosphonates that possess P-C-P bonds thus appear to have effects on the deposition of calcium phosphate *in vitro* and *in vivo* similar to those of inorganic pyrophosphate and the condensed phosphates, which possess P-O-P bonds. These phosphonates differ from the condensed phosphates in that they inhibit kidney calcification as well as aortic calcification and are active by mouth as well as parenterally. The wider spectrum of activity of the phosphonates *in vivo* may be due to the fact that they are more resistant to chemical and enzymic breakdown.

5. Phosphonates might be used therapeutically in man against diseases in which calcium salts deposit in soft tissues.

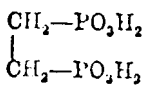
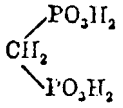
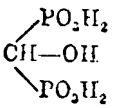
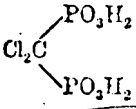
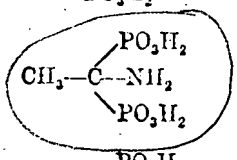
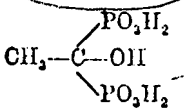
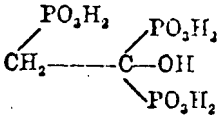
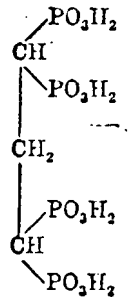
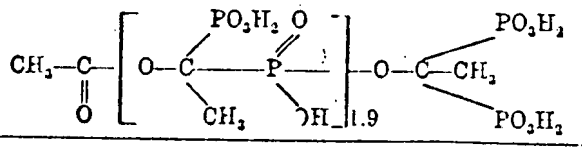
Key-words: Calcification, phosphonates, pyrophosphate, calcium, phosphorus, nephrocalcinosis, arteriosclerosis.

Low concentrations of inorganic pyrophosphate inhibit both the precipitation [1] and growth [2] of hydroxyapatite crystals *in vitro*. Since pyrophosphate has been found to be present in plasma at concentrations similar to those required to produce inhibition *in vitro* [3], it has been suggested that pyrophosphate might be one of the physiological agents responsible for preventing deposition of calcium phosphate in soft tissues *in vivo* [3, 4]. At sites of mineralization it was supposed that the pyrophosphate would have to be removed enzymically before deposition of calcium phosphate could occur.

In support of this hypothesis pyrophosphate and long chain condensed phosphates administered parenterally have been shown to be able to inhibit certain types of experimentally-induced calcification in animals. Thus these compounds prevent the aortic calcification induced by vitamin D_3 in rats [5] and skin calcification induced by dihydrotachysterol in rats [6, 7]. At the same doses they are, however, unable to inhibit the kidney calcification produced by vitamin D_3 [6], and have no effect upon the mineralization of bone [8]. It was thought that the differing sensitivity of various tissues to inhibition of calcification by condensed phosphates might be related to the amount of pyrophosphatase they contained; tissues such as kidney and bone that are rich in pyrophosphatases and alkaline phosphatases, enzymes which are now recognized to be able to act as pyrophosphatases [9—12], would be capable of hydrolysing the pyrophosphate before it could act. Since the intestine contains a high amount of alkaline phosphatase [13], a similar explanation might account for the observation that the condensed phosphates only inhibit calcification when administered subcutaneously but not when given orally.

Since the condensed phosphates were ineffective by mouth and did not act against all forms of soft tissue calcification their potential clinical application against disorders of calcification seemed somewhat limited. What seemed to be required were compounds similar to pyrophosphate in their behaviour towards hydroxyapatite but resistant to hydrolysis by pyrophosphatase. Compounds that possess the P-C-P bond instead of the P-O-P bond exhibit these properties [14—16]. In this paper we report the effect of a number of phosphonates possessing C-P or P-C-P bonds on the precipitation and crystallization of hydroxyapatite *in vitro* and on the calcification of rat aorta and kidney *in vivo*. The results indicate that the diphosphonates have a wider spectrum of activity than the condensed phosphates. Thus, not only are they active against aortic calcification but also against renal calcification and when given by mouth. Preliminary results have been presented elsewhere [15, 16].

Table 1. *List of phosphonates studied*

Name of compound	Abbreviation	Formula (as acid)
n-pentanemonophosphonic acid	PMP	$CH_3(CH_2)_4-PO_3H_2$
ethane-1:2-diphosphonic acid	E-1:2-DP	
methylenediphosphonic acid	MDP	
methylenehydroxydiphosphonic acid	MHDP	
dichloromethylenediphosphonic acid	Cl_2MDP	
ethane-1-amino-1:1-diphosphonic acid	EADP	
ethane-1-hydroxy-1:1-diphosphonic acid	EHDP	
ethane-1-hydroxy-1:1:2-triphosphonic acid	E-1-HTP	
propane-1:1:3:3-tetraphosphonic acid	PTeP	
polyester chain condensate of EHDP with acetic anhydride	Condensate I	

Materials and Methods

The phosphonates were synthesized by the Procter and Gamble Co., Cincinnati, Ohio, U.S.A., and were kindly supplied to us by Dr. M. D. Francis. Sodium salts of the compounds listed in Table 1 were used.

Effect of Phosphonates on the Formation of Apatite Crystals in vitro. The minimum product (formation product), [Ca] × [P], required to induce precipitation of calcium phosphate from solution was measured as described previously [1]. With this method 1.7 mM Ca is incubated with increasing amounts (0.5—8.0 mM) of phosphate in siliconized vessels on a shaker for 3 days at 37 °C. The solutions are buffered at pH 7.4 with 0.01 M barbital and the ionic strength is brought to 0.16 by adding the appropriate amount of KCl. After 3 days any crystals formed are removed by filtration through "Göttingen Membran" filters (0.45 μ pore size) and the amounts of calcium and phosphate

Table 2. *Influence of various phosphonates on the minimum product [Ca] × [P] in mM² (formation product) necessary to induce precipitation of calcium phosphate from solution. Incubation for three days at pH 7.4 and 37° C. Control values obtained from 11 incubation without phosphonates were 4.84 ± 0.12 (mean ± SE, [Ca] × [P] in mM²). The results shown here represent the increase in minimum [Ca] × [P] product observed in the presence of the phosphonate compared with the minimum [Ca] × [P] product required for crystal formation in a parallel experiment but in the absence of the phosphonate. Number of experiments in parentheses*

Compound	Mean increase in formation product over control value		
	Concentration of phosphonate		
	10^{-5} M	10^{-6} M	10^{-7} M
PMP	0.38 (6)	0.47 (8)	—
E-1:2-DP	0.50 (1)	0.20 (1)	—
MDP	—	2.21 (7)	0.62 (5)
MHDP	—	3.10 (1)	0.40 (1)
Cl_2MDP	5.30 (1)	1.10 (1)	—
EADP	—	4.90 (1)	0.30 (1)
EHDP	—	>7.30 (6)	1.88 (5)
E-1-HTP	0.70 (1)	−0.10 (1)	—
PTeP	0.20 (1)	−0.50 (1)	—
Condensate I	—	1.90 (1)	0.70 (1)

Table 3. The survival proportions of the untreated control group and the vitamin D_3 control, 17/20 and 53/60, are almost identical. Clearly there is no evidence that treatment with vitamin D_3 itself caused an increase in mortality. The survival proportions for each treatment group were compared with that for vitamin D_3 control using the two-sided t test. There was evidence (significant at the 1% and 5% level respectively) that MHDP and MDP, both at 10 mg P/kg s.c., caused an increase in mortality. A survival proportion of 6/10 differs from one of 53/60 at the 9% significance level. There is therefore a suggestion that EADP and E-1-HTP, at 10 mg P/kg s.c., may also cause an increase in mortality.

The only effect of the phosphonates on the body weight was that the animals who received 10 mg s.c. of MDP, EADP, EHDP, Condensate I, E-1-HTP and PTeP weighed less at the end of the experiment than the animals given Vitamin D alone. The s.c. administration of 1 mg P/kg and the oral administration had no effect.

Aorta Calcium. All the phosphonates except PMP had some effect in reducing aortic calcium when given at 10 mg P/kg s.c. (Table 3). All compounds except PMP, E-1-HTP and Condensate I were also effective at 1 mg P/kg s.c. Fewer compounds were effective by the oral route; MDP, Cl_2MDP, EHDP, E-1-HTP and Condensate I were effective at 10 mgP/kg p.os., whereas only MDP and perhaps PTeP were effective at 1 mg P/kg p.os.

Kidney Calcium. Seven of the ten phosphonates tested caused significant reductions in kidney calcium (Table 4). All of the significant differences listed in Table 4 can reasonably be interpreted as indicating real treatment effects, but there are two anomalies. The reductions in mean calcium when both MHDP and EADP were given at 10 mg P/kg s.c. were not significant whereas the reductions with 1 mg P/kg s.c. were in both cases highly significant. With MHDP at 10 mg P/kg given p.os there was also a highly significant reduction. As only 2 and 3 determinations respectively are available in the 10 mg P/kg s.c. treatment groups the evidence on balance favours the conclusion that these two treatments are both active.

In conclusion, there was evidence that the following compounds caused some inhibition of kidney calcification: MDP, MHDP, Cl_2MDP, EADP, EHDP, PTeP and Condensate I.

Table 3. *Influence of various phosphonates on the amount of calcium in the aortas of rats that received 75,000 IU vitamin D_3 per kg body weight daily for 5 days. Aorta calcium as mean $\log_{10}$ (mg Ca/g dry weight) ± SE of mean. Number of determinations in parentheses*

Compound	1 mg P/kg s.c.	Survival	1 mg P/kg p. os	Survival	10 mg P/kg s.c.	Survival	10 mg P/kg p. os	Survival
PMP	4.44 ± 0.19 (9)	10/10	4.57 ± 0.19 (9)	9/10	4.59 ± 0.19 (9)	10/10	4.66 ± 0.20 (8)	9/10
E-1:2-DP	4.13 ± 0.21[b] (7)	9/10	4.39 ± 0.21 (7)	8/10	3.75 ± 0.19[c] (9)	10/10	4.45 ± 0.20 (8)	9/10
MDP	4.16 ± 0.20[b] (8)	10/10	3.90 ± 0.18[b] (10)	10/10	3.11 ± 0.28[c] (4)	4/10	3.17 ± 0.19[c] (9)	10/10
MHDP	3.62 ± 0.21[c] (7)	9/10	4.58 ± 0.21 (7)	9/10	3.66 ± 0.56[a] (1)	2/10	4.47 ± 0.23 (6)	9/10
Cl_2MDP	3.38 ± 0.19[c] (9)	9/10	4.51 ± 0.18 (10)	10/10	3.39 ± 0.21[c] (7)	9/10	3.25 ± 0.21[c] (7)	10/10
EADP	3.79 ± 0.19[c] (9)	9/10	4.42 ± 0.19 (9)	10/10	3.56 ± 0.25[c] (5)	6/10	4.57 ± 0.19 (9)	10/10
EHDP	3.95 ± 0.18[c] (10)	10/10	4.33 ± 0.23 (6)	7/10	3.33 ± 0.19[c] (9)	9/10	3.38 ± 0.23[b] (6)	10/10
E-1-HTP	4.70 ± 0.20 (8)	9/10	4.58 ± 0.19 (9)	10/10	4.05 ± 0.23[b] (6)	6/10	4.25 ± 0.19[a] (9)	10/10
PTeP	4.22 ± 0.20[b] (8)	10/10	4.23 ± 0.19[a] (9)	10/10	3.88 ± 0.21[c] (7)	7/10	4.41 ± 0.18 (10)	10/10
Condensate I	4.42 ± 0.23 (6)	6/10	4.82 ± 0.19 (9)	10/10	3.52 ± 0.19[c] (9)	9/10	4.26 ± 0.19[a] (9)	10/10

Vitamin D_3 only: 4.64 ± 0.03 (51)
Untreated control: 3.31 ± 0.14 (10)
} difference significant at 1% level. Survival { 53/60 / 17/20

[a] Denotes that difference in mean aortic calcium compared with vitamin D_3 Control is significant at 10% level.
[b] Denotes that difference in mean aortic calcium compared with vitamin D_3 Control is significant at 5% level.
[c] Denotes that difference in mean aortic calcium compared with vitamin D_3 Control is significant at 1% level.

remaining in solution are compared with those present at the beginning of the incubation, thus making it possible to determine the minimum [Ca] × [P] product required to induce precipitation.

Prevention of Calcification of Aortas and Kidneys in Rats. Calcification of aortas and kidneys was induced in female Wistar rats, weighing 150—200 g, by giving them 75,000 IU (1.875 mg) vitamin D_3/kg body wt. per day by mouth for five days. The experimental procedure was similar to that described previously [5]. The phosphonate compounds were adjusted to pH 7.4 and were given daily at doses of 1 or 10 mg P/kg body wt., subcutaneously (s.c.) or orally (p.os), from two days before starting vitamin D_3 up to eight days after the end of administration of vitamin D_3, when the animals were killed. The concentrations of the compounds were such that the daily dose was given subcutaneously or orally in 0.2 ml/100 g body wt. Each treatment group contained 10 animals. There were two control groups: one consisted of 60 animals that received vitamin D_3 but instead of phosphonates received daily subcutaneous injections of 0.2 ml/100 g body wt. of 0.9% (w/v) NaCl, the other consisted of 20 animals that received no treatment whatsoever. All treatments were allocated to individual animals at random and each cage contained five animals. All animals were fed a standard diet (No. 194 from Nafag, Gossau, Switzerland).

In order to see whether or not the phosphonates had any effect on the hypercalcaemia induced by vitamin D_3, venous blood was collected from the orbit of each animal 24 hours after the last dose of the vitamin, when the hypercalcaemia due to the vitamin should be at its peak [5]. After sacrifice the aortas and kidneys were removed and their weights were determined after drying to constant weight at 105 °C. The organs were then ashed in a muffle oven at 600 °C for eight hours. The residues were dissolved in 0.5 N-HCl and the calcium determined. The calcium of the aortas and plasma was determined by photometric titration with EDTA using calcichrome as an indicator [17] and the calcium of the kidneys by atomic absorption spectroscopy (Perkin Elmer Model 290 B).

Statistical Analyses. The differences between the vitamin D_3 group and each treatment group (Tables 3, 4 and 5) were examined statistically for evidence of treatment effects, i.e. a difference in mean calcium content compared with vitamin D_3 control. In the case of aorta and kidney calcium the values in each treatment group followed a skewed distribution so the logarithms of the calcium values were used for statistical analysis. It was assumed throughout that the variation between animals receiving the same treatment was not dependent on the treatment. The standard errors of treatment means were calculated from a pooled estimate of the variance, and the significance of differences in mean compared with vitamin D_3 control determined by two-sided *t* tests.

Since the primary interest lies in detecting compounds which inhibit calcification, i.e. in compounds that cause a reduction in mean calcium compared with vitamin D_3 control, a one-tailed test would be more appropriate. However, the possibility that a compound may cause an increase in mean calcium should also be investigated and for this reason the results of two-tailed tests have been given. In fact there were only two significant increases in mean calcium, with PMP and E-1:2-DP both on plasma. Neither of these, when studied with the other plasma calcium results for these compounds, is regarded as sufficient evidence for a real treatment effect.

Significant reductions in mean calcium were not automatically interpreted as indicative of treatment activity. The choice of the 5% significance level, although customary, is nevertheless arbitrary. Also as 120 t tests were performed some reductions are expected to achieve significance by chance alone. An additional criterion was therefore used in forming conclusions about treatment activities. It was expected that, if a compound was active in inhibiting calcification, 10 mg/kg doses should be more active than 1 mg/kg doses and that applications given s.c. should be more active than applications given p.os. We felt justified in interpreting significant reductions as evidence for treatment activity if these constraints were satisfied. This applies also to differences significant at the 10% level only provided they were reinforced by significant differences in other applications of the same compound. The fact that these constraints were satisfied in all but three cases (PTeP on aorta calcium, MHDP and EADP on kidney calcium) gives reassuring support to the validity of the whole experiment.

Results

Effect of Phosphonates on the Precipitation of Calcium Phosphate in vitro. Table 2 shows the effect of the phosphonates on the minimum product, [Ca] × [P], required to form crystals *in vitro*. EHDP appeared to be the most effective inhibitor of precipitation and exerted a strong effect at 10^{-5} M. EHDP, EADP, MHDP, MDP, Condensate I and Cl_2MDP were all good inhibitors at 10^{-6} M. The remaining compounds, E-1-HTP, E-1:2-DP, PMP and PTeP had only minimal effects. At concentrations greater than 10^{-4} M the calcium salts of some of the phosphonates precipitated from solution.

Effect of the Phosphonates on Vitamin D_3-induced Calcification of Aortas and Kidneys and on Plasma Calcium. Tables 3, 4 and 5 summarize the effect of the various phosphonates on the calcium content of aortas, kidneys and plasma of rats treated with vitamin D_3. Vitamin D_3 significantly increased the calcium content of aortas, kidneys and plasma of animals treated with vitamin D_3 alone compared with untreated control animals. The numbers of animals surviving the duration of the experiment are shown in

Table 4. *The influence of various phosphonates on the amount of calcium in the kidneys of rats that received 75,000 IU vitamin D_3 per kg body weight daily for 5 days. Kidney calcium as mean $\log_{10}$ (mg Ca/g dry weight) ± SE of mean. Number of determinations in parentheses*

Compound	1 mg P/kg s.c.	1 mg P/kg p. os	10 mg P/kg s.c.	10 mg P/kg p. os
PMP	3.81 ± 0.19 (5)	4.03 ± 0.14 (9)	3.83 ± 0.16 (7)	3.94 ± 0.15 (8)
E-1:2-DP	3.71 ± 0.19 (5)	3.83 ± 0.17 (6)	3.93 ± 0.17 (6)	3.82 ± 0.16 (7)
MDP	3.16 ± 0.17[b] (6)	3.71 ± 0.16 (7)	3.28 ± 0.19[b] (5)	3.23 ± 0.19[b] (5)
MHDP	3.11 ± 0.16[b] (7)	3.96 ± 0.16 (7)	3.66 ± 0.31 (2)	2.30 ± 0.17[b] (6)
Cl_2MDP	3.18 ± 0.17[b] (6)	3.41 ± 0.16[b] (7)	2.97 ± 0.14[b] (9)	2.99 ± 0.13[b] (10)
EADP	3.23 ± 0.17[b] (6)	3.99 ± 0.15 (8)	3.85 ± 0.24 (3)	3.61 ± 0.13 (10)
EHDP	3.36 ± 0.15[b] (8)	3.77 ± 0.17 (6)	3.12 ± 0.17[b] (6)	3.02 ± 0.17[b] (6)
E-1-HTP	3.69 ± 0.16 (7)	3.96 ± 0.13 (10)	3.77 ± 0.17 (6)	3.55 ± 0.17 (6)
PTeP	3.70 ± 0.24 (3)	3.89 ± 0.16 (7)	3.14 ± 0.19[a] (5)	4.01 ± 0.19 (5)
Condensate I	3.54 ± 0.17 (6)	3.95 ± 0.15 (8)	3.24 ± 0.19[b] (5)	3.80 ± 0.17 (6)

Vitamin D_3 only: 3.83 ± 0.07 (37)
Untreated control: 2.73 ± 0.11 (15)
} difference significant at 1% level.

[a] Denotes that difference in mean compared with vitamin D_3 Control is significant at 5% level.
[b] Denotes that difference in mean compared with vitamin D_3 Control is significant at 1% level.

Table 5. *Influence of various phosphonates on the plasma calcium of rats that received 75,000 IU vitamin D_3 per kg body weight daily for 5 days. The values were taken 24 hours after the last dose of vitamin D_3. Plasma calcium as mg per 100 ml ± SE of mean. Number of determinations in parentheses*

Compound	1 mg P/kg s.c.	1 mg P/kg p. os	10 mg P/kg s.c.	10 mg P/kg p. os.
PMP	15.90 ± 0.54[c] (9)	13.51 ± 0.57 (8)	14.29 ± 0.57 (8)	14.79 ± 0.54 (9)
E-1:2-DP	14.51 ± 0.61 (7)	14.53 ± 0.66 (6)	15.61 ± 0.57[a] (8)	14.66 ± 0.54 (9)
MDP	15.02 ± 0.54 (9)	14.41 ± 0.54 (9)	11.82 ± 0.57[c] (8)	14.04 ± 0.54 (9)
MHDP	14.36 ± 0.51 (10)	14.73 ± 0.61 (7)	14.09 ± 0.93 (3)	15.19 ± 0.61 (7)
Cl_2MDP	14.03 ± 0.66 (6)	15.08 ± 0.51 (10)	12.98 ± 0.66[b] (6)	12.94 ± 0.54[b] (9)
EADP	14.10 ± 0.66 (6)	14.68 ± 0.72 (5)	12.70 ± 0.61[b] (7)	14.63 ± 0.54 (9)
EHDP	14.80 ± 0.51 (10)	14.80 ± 0.66 (6)	13.07 ± 0.61[a] (7)	14.10 ± 0.54 (9)
E-1-HTP	14.76 ± 0.61 (7)	14.56 ± 0.51 (10)	14.62 ± 0.57 (8)	13.98 ± 0.66 (6)
PTeP	15.24 ± 0.61 (7)	14.82 ± 0.66 (6)	15.35 ± 0.66 (6)	15.50 ± 0.80 (4)
Condensate I	13.57 ± 0.80 (4)	14.79 ± 0.54 (9)	14.09 ± 0.57 (8)	14.78 ± 0.72 (5)

Vitamin D_3 only: 14.36 ± 0.25 (41)
Untreated Control: 10.84 ± 0.39 (17)
} difference significant at 1% level.

[a] Denotes that difference in mean compared with vitamin D_3 Control is significant at 10% level.
[b] Denotes that difference in mean compared with vitamin D_3 Control is significant at 5% level.
[c] Denotes that difference in mean compared with vitamin D_3 Control is significant at 1% level.

Plasma Calcium. Only 3 of the phosphonates, MDP, Cl_2MDP and EADP, showed evidence of being able to reduce the hypercalcaemia induced by vitamin D_3 (Table 5). These 3 compounds were effective when they were given at 10 mg P/kg s.c. Cl_2MDP was also effective at this dose when given p.os.

Discussion

These experiments show that various phosphonates act in a similar manner to pyrophosphate and condensed phosphates on the inhibition of crystallization of hydroxyapatite *in vitro* and on the prevention of aortic calcification in rats given large doses of vitamin D_3. Some of these phosphonates have additional properties not shared with the condensed phosphates; these include the ability to prevent aortic calcification when given orally as well as subcutaneously and the ability to prevent kidney calcification. Furthermore, in opposition to condensed phosphates [8], large doses of EHDP (greater than 2.5 mg P/kg) have been found to inhibit mineralisation of rat and dog bones and to produce a rachitic and osteomalacic type appearance on X-ray and by histology. No such lesions were observed with similar and higher doses of Cl_2MDP[1, 2]. The inhibition of bone mineralisation ceased and calcification resumed within four weeks after stopping injections of EHDP[1]. The fact that, unlike the polyphosphates, the phosphonates are effective orally and are able to inhibit kidney calcification may be attributable to their marked resistance to spontaneous and enzymic hydrolysis. However, as would be expected, most of the active compounds were less effective by mouth than subcutaneously, suggesting that their absorption from the intestinal tract may not be complete. This is consistent with other studies in rats which have shown that, in the case of ^{14}C-labelled EHDP, only about 3% of an oral load is absorbed[3].

1 King, W. R., Michael, W. R.: Personal communication.
2 Fleisch, H., Russell, R. G. G., Mühlbauer, R. C.: Unpublished observations.
3 Francis, M. D., personal communication.

When the effect of any apparent compound on aortic and kidney calcification at the various doses was considered, there was a fair correlation between the ability of any compound to inhibit crystal growth *in vitro* and its overall ability to inhibit calcification *in vivo*. This strengthens the view that the biological activity of these compounds could be a consequence of their observed action on crystal growth [14, 16]. In general, the compounds that have, so far, proved most active have contained the P-C-P bond. Since compounds that contain the P-C-P bond are related in structure to those that contain the P-O-P bond, the diphosphonates, pyrophosphate and polyphosphates may all act on crystal growth by similar mechanisms. In this study the three most effective inhibitors of calcification appeared to be MDP, Cl_2MDP and EHDP. The compounds PMP, which contains a single C-P bond, and E-1:2-DP, which contains a P-C-C-P bond, were less effective or inactive. It is not known whether any of the compounds are metabolized *in vivo* and to what extent this might alter their activity.

It is of interest that some of the diphosphonates (Cl_2MDP, MDP and EADP) can partially prevent the hypercalcaemia induced by vitamin D_3. This rise in plasma calcium after treatment with vitamin D_3 is probably the result of two processes, firstly an increase in intestinal absorption of calcium and secondly an increased resorption of bone. The ability of Cl_2MDP, MDP and EADP to reduce plasma calcium could be due to an interference with either of these processes. Alternatively it might be due to precipitation of circulating calcium as the insoluble calcium phosphonate. Since there is good evidence that these doses of phosphonates can inhibit bone resorption in living rats [15, 18, 19] it is probable that the effect on plasma calcium is at least partly attributable to inhibition of bone resorption.

It is clear, however, that any reduction in plasma calcium concentration cannot be the sole explanation of the ability of the phosphonates to reduce aortic and kidney calcification, since several compounds had no effect on plasma calcium, but were, nonetheless, good inhibitors of calcification. This is also true of the condensed phosphates which can inhibit calcification without changing plasma calcium [5]. One of the most effective inhibitors of calcification, namely EHDP, did not produce a hypocalcaemia but on the contrary a hypercalcaemia when tested shortly after its administration. The phosphonates do not seem to act either by an intestinal or kidney effect, since no diminished intestinal absorption or increased kidney excretion was seen in rats under balance studies [20]. It is possible, however, that the inhibitory effects of phosphonates on calcification may be due to other causes than crystal growth inhibition, for example, an inhibition of pyrophosphatases or inhibition of conversion of vitamin D_3 to its biologically more active 25-hydroxy metabolite. Our preliminary studies of the interaction of phosphonates with pyrophosphatases indicate that the phosphonates are relatively poor inhibitors of pyrophosphatases and act principally by the chelation of metal ions necessary for enzyme activity. The significance of possible inhibitory effects on pyrophosphatases *in vivo* cannot yet be assessed. The effects of these phosphonates on other metabolic processes in which pyrophosphate is involved are also not known.

The experiments carried out so far indicate that the diphosphonates might be used therapeutically in man against diseases that involve the abnormal deposition of calcium in soft tissues as previously suggested by Francis [14]. Preliminary experience in myositis ossificans in humans suggests that EHDP prevents the progress of the disease without disturbing the formation and mineralisation of normal bone [21].

Acknowledgements. This work has been supported by the Procter and Gamble Company, Cincinnati, U.S.A.

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Prof. Dr. H. Fleisch
Pathophysiologisches Institut
CH-3012 Berne, Hügelweg 2

Letters to the Editor

DIPHOSPHONATES IN THE TREATMENT OF MYOSITIS OSSIFICANS

SIR,—Myositis ossificans progressiva is a rare disease of childhood, characterised by progressive and usually irreversible ossification of soft tissues.[1] The cause is unknown and there is no recognised treatment. On the basis of various experimental studies [2-4] showing that disodium ethane-1-hydroxy-1, 1-diphosphate (E.H.D.P.) prevents the deposition of calcium phosphate both in vivo and in vitro, an investigation was begun to determine whether this compound favourably affects the progress of myositis ossificans.

We have so far treated three patients with this agent. Two were in an acute progressive stage, and one in a "stabilised" stage. Of the acute cases, one patient was 16 months old, had shown progressive symptoms for 4 months, and had considerable limitation of motor and respiratory function. The second was 3 years old, had suffered intermittent progressive attacks every second month for 2 years, and exhibited severe deformities and limitation of movement. During treatment of these two patients with E.H.D.P., at a daily dose of 10 mg. per kg. by mouth, most of the newly formed soft-tissue swellings regressed within a few days without any evidence of calcification on subsequent X-rays. This behaviour has not been observed in the normal evolution of such lesions. At the same time the range of joint movement improved. Furthermore, the progress of the disease has apparently been slowed or even halted, for 18 months in the first case, and for 12 months in the second. Both patients had acute exacerbations when treatment was stopped. In the first patient there has been some regression in regions of pre-existing ossification.

The third patient was a 14-year-old boy who had had progressive symptoms from birth till the age of 10 years, after which time the disease had progressed no further. 1 month of treatment produced no demonstrable beneficial effect on the extensive but stabilised lesions. No adverse effects were encountered during administration of E.H.D.P. in any of the patients.

The beneficial effects of E.H.D.P. on the two acute progressive cases of myositis ossificans progressiva have been clearly established. Further trials of this agent will continue in view of these encouraging results.

Columbia Presbyterian Medical Center, New York, N.Y., U.S.A. — C. A. L. BASSETT.

University Departments of Pædiatrics, Clinical Pharmacology, and Pathophysiology, Berne, Switzerland. — A. DONATH, F. MACAGNO, R. PREISIG, H. FLEISCH.

Procter & Gamble, Ltd., Cincinatti, Ohio, U.S.A. — M. D. FRANCIS.

PRENATAL DIAGNOSIS AND SELECTIVE ABORTION

SIR,—One cannot question the value of scientific studies of amniotic fluid and cells to further our knowledge of some of the inherited metabolic disorders. When, however, the main object of these studies is to encourage therapeutic abortions, one can certainly question this from the point of view of the unborn baby.

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One can understand how some parents, alarmed by information about a possibly defective pregnancy, would consider this form of antenatal euthanasia a logical solution. Surely, however, we should consider the position of the unborn baby. Scientific studies have shown that the fetus has all the necessary criteria of a live human being at eight weeks of gestation, or even earlier. Surely these small human beings have some right to life despite the mental stress placed on their parents. When parents are faced with the possibility of a defective baby, should they not either be strongly encouraged to prevent pregnancy, or else be prepared to accept a defective child. Admittedly, this argument may seem rather futile, particularly in Britain, when perfectly normal unborn babies are being destroyed simply because their parents do not want them.

One can also question the approach to selective abortion, since it is part of the process of lessening the respect for human life. A vivid illustration of this was described recently in the *British Medical Journal*.[1] In this situation a normal baby which had been aborted, survived briefly; and there were serious questions as to whether or not this baby had a right to live. Thus we see the subtle shift from antenatal to postnatal euthanasia.

Departments of Pædiatrics and Pathological Chemistry, University of Western Ontario. — L. L. DE VEBER.

SPEAKING AT MEDICAL MEETINGS

SIR,—The advice given by Dr. Meadow (Sept. 20, p. 631) is excellent. Many societies now send out printed recommendations along the same lines to all prospective speakers, however eminent, but these continue to be neglected. Even clear advice about the number of lines on a slide is disregarded. This summer, one man travelled from Asia Minor to New York to show twenty-five columns of black figures on a brown background.

But more common is the failure of a speaker to judge the ability of a particular audience to follow him. This should not be too difficult, for the majority of audiences are fairly homogeneous. At a recent symposium on a new drug, a research-worker from the U.S.A. spoke for fifteen minutes, and it was only in the last minute that I even appreciated that he had been talking about differences in enzyme systems in mammals and bacteria.

The organisers of meetings cannot be completely absolved from responsibility, since there is an increasing tendency to whittle down the time allowance and to believe that three ten-minute papers are better than one of thirty minutes. Recently at least one worm turned. At a combined meeting of a British and a Continental society, three quick papers with a multitude of complex flow-volume diagrams to an audience with physicians predominating led to a very legitimate protest from the floor—and this unenviable task was undertaken by an American visitor.

It seems to me that societies should take more steps to protect the customer. In journals we are protected to a considerable extent by the editor. With books, the reviewer gives us some guidance. But at a medical meeting we are completely at the mercy of the speaker. A society cannot very well ask to see a complete paper beforehand, and what may be satisfactory in print may be very different when spoken or read by some people. I would suggest that societies should appoint one capable and intelligent member of the audience to prepare a critical review of the presentation which should then be sent to the contributor. This will not save that particular audience, but, in these days when so many people read similar papers all over the world, it should help to protect others from similar injury in the future.

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Calc. Tiss. Res. 3, 151—162 (1969)

The Inhibition of Calcium Hydroxyapatite Crystal Growth by Polyphosphonates and Polyphosphates

MARION D. FRANCIS

Miami Valley Laboratories, The Procter & Gamble Company, Cincinnati, Ohio

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The formation of crystalline calcium hydroxyapatite from solutions of calcium and phosphate ions and the inhibition of calcium hydroxyapatite crystal growth by polyphosphonates and polyphosphates have been studied. The polyphosphonates, disodium ethane-1-hydroxy-1,1-diphosphonate and disodium dichloromethane diphosphonate, are effective inhibitors of calcium hydroxyapatite crystal growth. The polyphosphates are also effective inhibitors of calcium hydroxyapatite crystal growth as long as the required level of intact polyphosphate is present in the system. However, because of their hydrolytic instability, which is enhanced by high temperature, low pH, and certain enzymes, the concentration of the polyphosphate decreases with time *in vitro*, and its activity as an inhibitor is lost. In contrast to the polyphosphates, the polyphosphonates are hydrolytically stable. The polyphosphonates are chemisorbed on the surface of the microcrystallites of calcium hydroxyapatite and, in the manner of other known crystal growth poisons, thus prevent further crystal growth. The stability of the polyphosphonates and their chemisorption on apatite suggest their use in medical and dental applications involving pathological calcium and phosphate metabolism.

Key words: Calcification — Physiologic — Phosphonic Acids — Phosphates — Crystallization — Electron Microscopy.

On a étudié la formation de l'hydroxyapatite de calcium cristallin à partir de solutions d'ions de calcium et de phosphate et l'inhibition de la croissance de cristaux de l'hydroxyapatite de calcium au moyen de polyphosphonates et de polyphosphates. Les polyphosphonates, éthane hydroxy-1-diphosphonate-1,1 de disodium et dichlorométhanediphosphonate de disodium, sont inhibiteurs efficaces contre la croissance de cristaux de l'hydroxyapatite de calcium. Les polyphosphates sont aussi inhibiteurs efficaces contre la croissance de cristaux de l'hydroxyapatite de calcium tant que le niveau exigé de polyphosphate intact est présent dans le système. Cependant, à cause de leur instabilité hydrolytique, qui est soulignée par une température élevée, valeur de pH basse, et certaines enzymes, la concentration du polyphosphate diminue avec le temps *in vitro*, et son activité comme inhibiteur est perdue. Au contraire aux polyphosphates, les polyphosphonates sont hydrolytiquement stables. Les polyphosphonates sont chimiosorbés sur la surface des microcristallites de l'hydroxyapatite de calcium, ainsi empêchant l'occurrence d'autre croissance de cristaux semblable à l'action d'autres poisons connus de croissance de cristaux. On propose l'extension de cette action sur la formation de l'apatite et cette stabilité des polyphosphonates aux applications médicales et dentaires concernant le metabolisme pathologique de calcium et de phosphate.

Die Bildung des kristallinen Calciumhydroxyapatit aus Lösungen, welche Calcium- und Phosphationen enthalten, und die Hemmung der Bildung von kristallinen Calciumhydroxyapatit durch Polyphosphonate und Polyphosphate wurden untersucht. Polyphosphonate,

For reprints: Dr. MARION D. FRANCIS, Miami Valley Laboratories, The Procter & Gamble Company, P.O. Box 39175, Cincinnati, Ohio 45239.

Dinatriumäthan-1-hydroxyl-1,1-diphosphonat und Dinatriumdichloromethandiphosphonate verhindern das Kristallwachstum des Calciumhydroxyapatits. Die Polyphosphate verhindern ebenfalls das Kristallwachstum des Calciumhydroxyapatits, solange die notwendige Konzentration des nicht hydrolysierten Polyphosphats vorhanden ist. Wegen ihrer hydrolytischen Unbeständigkeit, die durch hohe Temperatur, niedrige pH und bestimmte Enzyme erhöht wird, vermindert sich jedoch die Konzentration des Polyphosphats allmählich *in vitro*, und ihre Hemmungsaktivität geht verloren. Im Gegensatz zu den Polyphosphaten sind die Polyphosphonate hydrolytisch beständig. Die Polyphosphonate werden an der Oberfläche der Mikrokristallite des Calciumhydroxyapatits chemisorbiert und verhindern, wie andere bekannte Kristallwachstumsgifte, auf diese Weise weiteres Kristallwachstum. Die Beständigkeit der Polyphosphonate und ihre Chemisorption an dem Apatit empfehlen ihren Gebrauch in der ärztlichen und zahnärztlichen Praxis, soweit sie den pathologischen Calcium- und Phosphatstoffwechsel betreffen.

Introduction

The formation of calcium phosphate solids is of fundamental importance to man because calcium phosphate in the form of calcium hydroxyapatite (HA) is the main constituent of the skeletal system. Furthermore, the deposition of calcium phosphate, again primarily as HA, in regions of the body which should not calcify, is encountered in many pathological conditions such as dental calculus, bursitis, arthritis, and many other forms of ectopic calcification (Selye, 1962). Numerous workers (Brown, 1966; MacGregor and Brown, 1965; Eanes *et al.*, 1965; Glimcher, 1965; and Walton *et al.*, 1967) have investigated the mechanism of formation of HA or bone mineral under solution conditions, but there is no complete agreement on the route by which calcium and orthophosphate ultimately become the crystalline material called hydroxyapatite. There is general agreement, however, on the fact that when calcium and orthophosphate are mixed together at high pH the solid formed is initially amorphous, as defined by a lack of ability to obtain diffraction patterns by the physical methods of X-ray and electron diffraction. The latter technique suggests the lack of organized atomic layers of greater than about 2 to 3 unit cells (Bienenstock and Posner, 1968). The role of pyrophosphate, tripolyphosphate, and long chain phosphates in inhibiting the formation of HA *in vitro* and *in vivo* has been clearly shown by Fleisch and co-workers (Fleisch and Neuman, 1961; Fleisch *et al.*, 1965; Irving *et al.*, 1966; Schibler and Fleisch, 1966; and others, Newesely, 1967; Tuchweber and Gabbiani, 1967). The purpose of this paper is to show that polyphosphates and polyphosphonates inhibit specifically the crystal growth of HA and to show that the effects of these two materials on HA crystal growth differ by virtue of differences in their hydrolytic stability. This fundamental difference between the polyphosphates which hydrolyze and lose their ability to inhibit crystal growth of HA and polyphosphonates which do not, suggests the medical and dental application of the polyphosphonates to the treatment of pathological calcium and phosphate metabolism.

Methods

A stock solution of calcium chloride was made from $CaCl_2 \cdot 2H_2O$; the orthophosphate solution was made from $NaH_2PO_4 \cdot H_2O$. Equal volumes of the solutions were mixed together with agitation under nitrogen and were kept at constant pH, 7.4 or 12.0 by means of a Radio-

don Co., Westlake, Ohio) until time for recovery of the precipitate. The solutions containing precipitated calcium phosphate were filtered through 0.45 μ Millipore filters and the precipitates were air dried and analyzed or were embedded in methylmethacrylate, sectioned, and examined by electron microscopy and electron diffraction in a Siemens Elmiskop I (Siemens und Halske, Aktiengesellschaft, Wernerwerk für Meßtechnik, Karlsruhe, Germany; Siemens American, New York City). When materials were added to inhibit the crystal growth of hydroxylapatite, they were usually added to the phosphate solution, then the calcium solution was added to produce instantaneous precipitation, except where indicated otherwise. Concentrations of solutions and reaction conditions are listed in the legends to the figures. Calcium analysis was performed with a Perkin-Elmer, Model 303 atomic absorption spectrometer (Perkin-Elmer, Norwalk, Conn.). Phosphate was determined by various combinations of three methods depending upon whether only orthophosphate (Martin and Doty, 1949), combined ortho- and polyphosphate (Lucena-Conde and Prat, 1957), or total phosphorus (ortho- plus phosphonate) was desired. In the latter case, the samples were mixed with $KHSO_4$ and fused until the melt solidified; the latter was then taken into solution with acid, as usual, and analyzed for combined ortho- and polyphosphate (Lucena-Conde and Prat, 1957).

The inhibitors of crystal growth used were tetrasodium pyrophosphate decahydrate, pentasodium tripolyphosphate, disodium ethane-1-hydroxy-1,1-diphosphonate (EDHP), and disodium dichloromethanediphosphonate (Cl_2MDP). EHDP-1-^{14}C was synthesized from acetate-1-^{14}C by the method of Quimby and Prentice (1966) and was used in adsorption studies.

Results

When calcium and orthophosphate solutions are mixed together and held at constant pH of 7.4 for 45 minutes at 25°C, the precipitate formed is crystalline HA as shown by the electron diffraction pattern (Fig. 1a). The crystals formed are needle-like as shown in the electron micrograph (Fig. 1b). Some of the gel-like spheres originally reported by Weber *et al.* (1967) are still seen, however (Fig. 1b). If the same experiment is repeated but sodium pyrophosphate or sodium tripolyphosphate is added to the orthophosphate solution prior to addition of the calcium chloride, no crystallites are formed and the entire mass of solid precipitate is amorphous by electron diffraction (Fig. 2a). This precipitate has the appearance of aggregated clusters of small spheres (Fig. 2b) similar to the initial amorphous form (Weber *et al.*, 1967). In another experiment the control calcium and phosphate solutions at pH 7.4 were mixed and filtration was begun immediately. The electron diffraction pattern (Fig. 3a) obtained from the precipitate was a good crystal pattern of hydroxyapatite and the crystallite particles were readily visible by electron microscopy (Fig. 3b). Crystals in this control system were not as large as the crystals shown in Fig. 1b because the precipitate was filtered immediately rather than after 45 minutes of incubation. If EHDP is added to a similar system (in the phosphate solution), crystal growth of HA is prevented, the precipitate formed is amorphous by electron diffraction techniques (Fig. 3c), and the precipitate has an agglomerated gel-like appearance (Fig. 3d).

The important difference between the polyphosphonates and the polyphosphates with regard to their interference with the crystal growth of HA lies in the hydrolytic stability of the polyphosphonates and the hydrolytic instability of the polyphosphates. This difference is illustrated by the data shown in Fig. 4 (a, b, c, d, e, f). In this series of experiments the calcium phosphate precipitates were formed at pH 7.4 in the presence of either pyrophosphate, tripolyphosphate,

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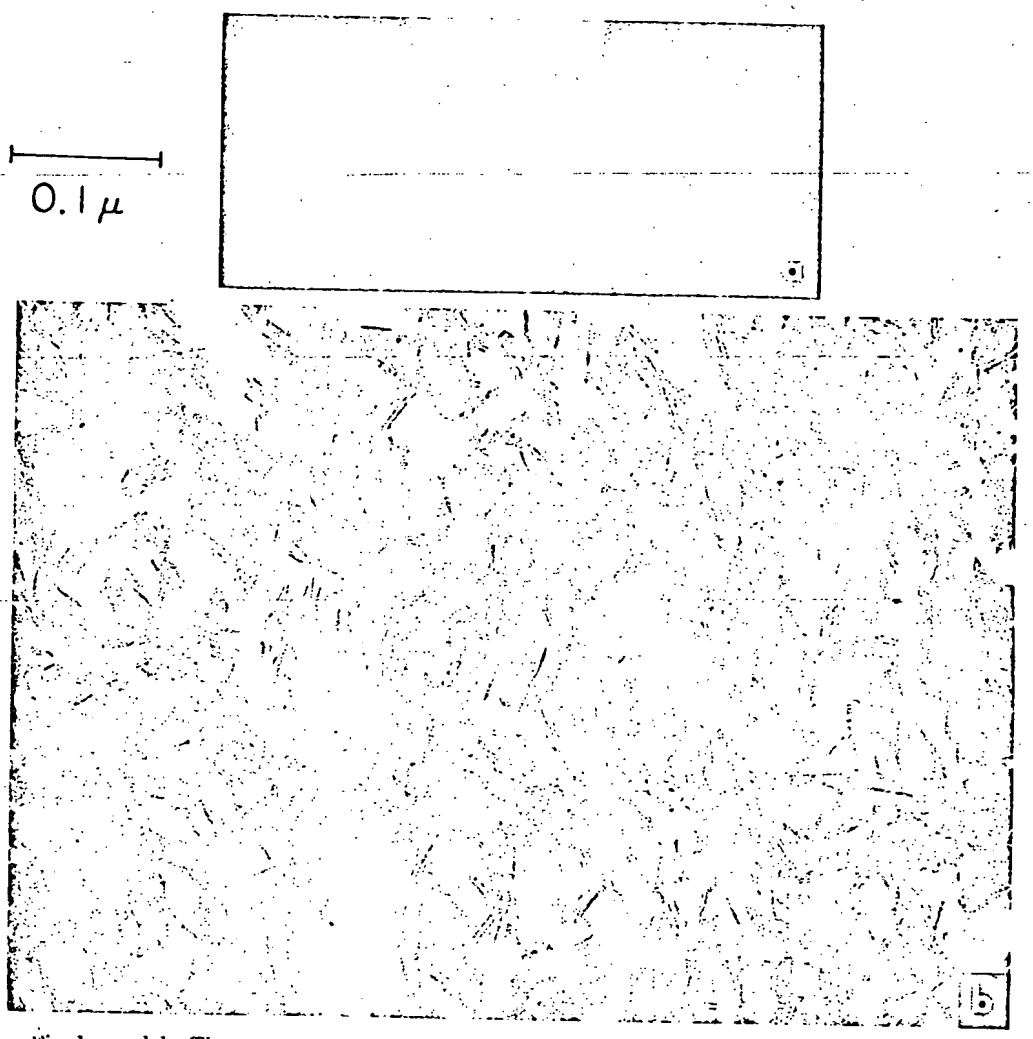

Fig. 1a and b. The appearance of freshly precipitated calcium orthophosphate. a. Electron diffraction pattern of hydroxyapatite. The precipitate was formed by reaction of 1×10^{-2} M $CaCl_2$, 1×10^{-2} M NaH_2PO_4, pH = 7.4, 45 minutes at 25°C. b. Electron micrograph of precipitate from (a) showing the long needle-like crystals of hydroxyapatite (×190,000)

48 hours to facilitate hydrolysis. In the control, no inhibitor, a good but spotty pattern for HA was obtained by electron diffraction (Fig. 4a). The large rectangular crystals responsible for this pattern are seen in the micrograph, Fig. 4b. Fig. 4c shows the diffraction pattern obtained from the precipitate formed in the presence of pyrophosphate; as in the control, a good but spotty pattern for HA was obtained indicative of the presence of large crystals as shown in the micrograph, Fig. 4d. Thus, it is obvious that while pyrophosphate is an excellent inhibitor of HA crystal growth, under the conditions of this experiment it is destroyed by hydrolysis and HA crystals are then free to form without interference. When tripolyphosphate was added to the

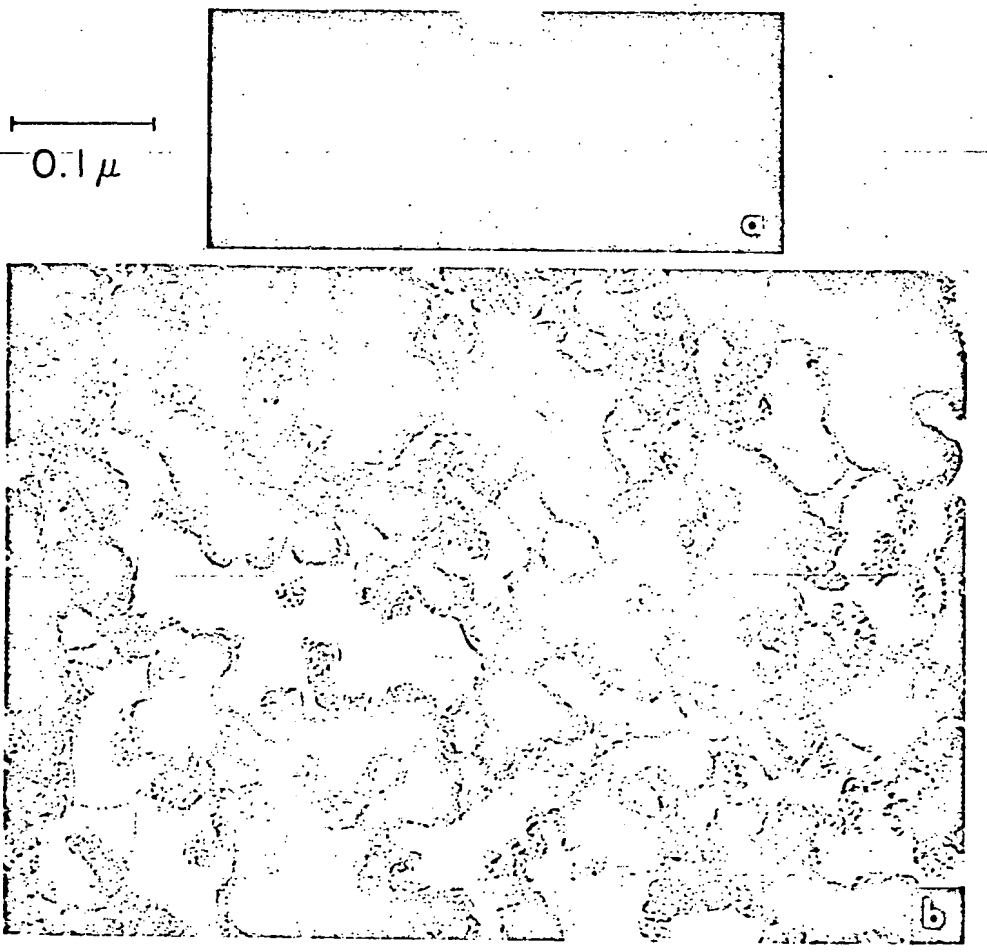

Fig. 2a and b. The appearance of freshly precipitated calcium orthophosphate inhibited by tripolyphosphate ion. a. Amorphous electron diffraction pattern. The precipitate was formed by reaction of 1×10^{-2} M $CaCl_2$, 1×10^{-2} M NaH_2PO_4, 1×10^{-3} M $Na_5P_3O_{10}$, pH 7.4, 45 minutes at 25°C. b. Electron micrograph of gel-like agglomerates of calcium phosphate (×190,000)

those with pyrophosphate shown in Fig. 4c, d. When calcium phosphate was precipitated in the presence of EHDP, however, there was no evidence of crystal formation, even after 48 hours incubation at 80°. The electron diffraction pattern for this precipitate shows it to be amorphous (Fig. 4e) indicating no organized atomic (or ionic) arrangements within the resolving power of this technique. The electron micrograph (Fig. 4f) also shows no indication of crystallinity, thus EHDP was totally effective in inhibiting the crystal growth of HA. When Cl_2MDP was added to the system the results were identical to those with EHDP as shown in Fig. 4e, f.

In order to investigate the nature of the inhibition of HA crystallization, two

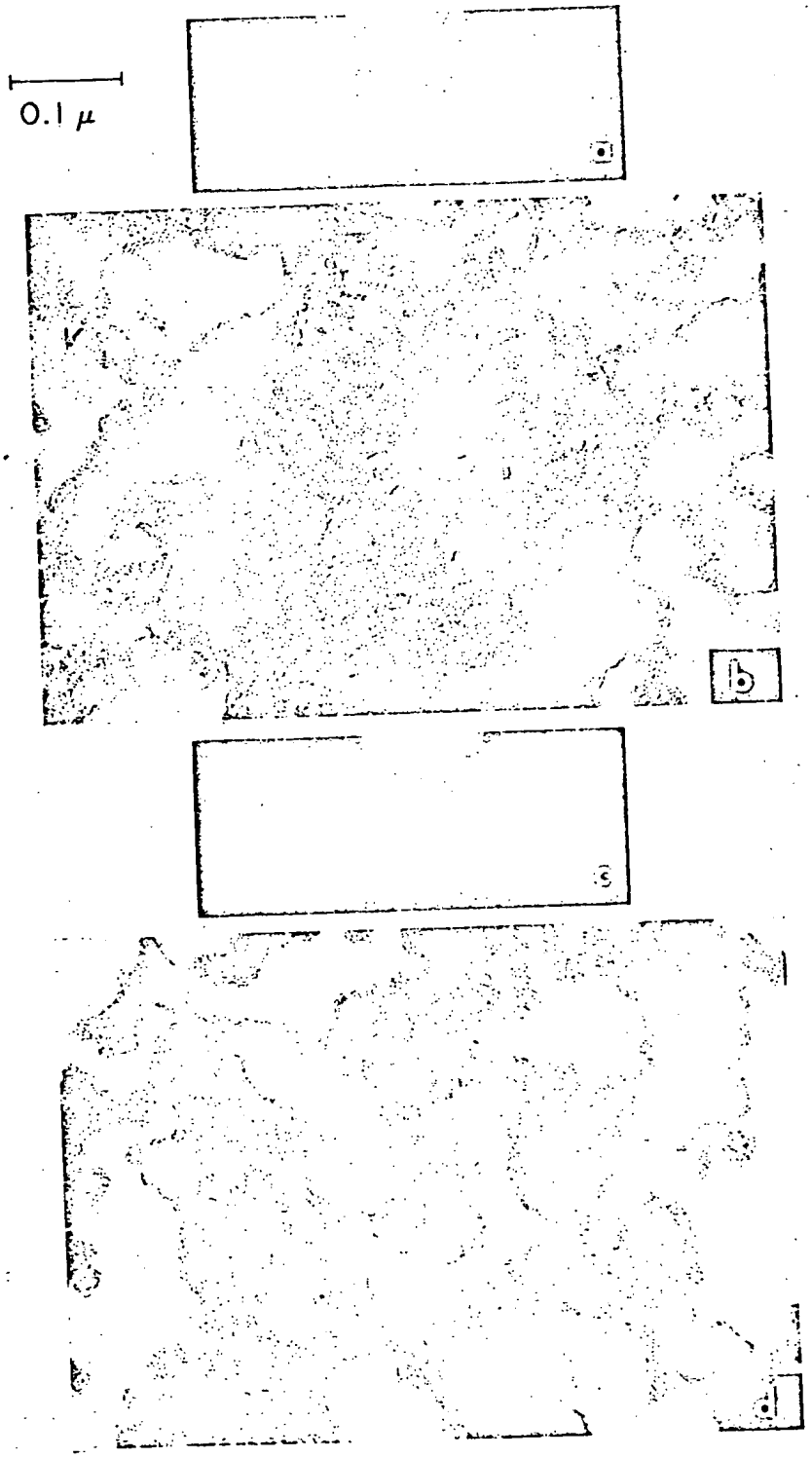

Fig. 3a—d

in Fig. 5. Calcium phosphate, Precipitate-1, from the control (with no EHDP) and similar crystallographically to precipitate in Fig. 3a, b was placed with Filtrate-2 (the filtrate from the calcium phosphate precipitated in the presence of EHDP). Large crystals were obtained after 48 hour incubation (similar to those seen in Fig. 4b), and a spotty HA pattern was obtained indicating that the Filtrate-2 did not contain sufficient EHDP to affect crystal growth (less than 1×10^{-5} M). In the other possible combination, Precipitate-2 (the calcium phosphate precipitate in the presence of EHDP) and similar to precipitate in Fig. 3c. d, was combined with Filtrate-1 from the control. In this case a stable colloid was formed in 8 to 16 hours and after 48 hours crystal growth of HA was still almost completely inhibited. In this experiment crystallites of HA from Precipitate-2 incubated in Filtrate-1 did grow sufficiently to give a HA electron diffraction pattern. Electron microscopy, however, failed to show any distinct crystallites (the particles were similar to those shown in Fig. 4f). Thus, EHDP was principally associated, coprecipitated, or occluded with Precipitate-2, not with Filtrate-2.

To understand further the association of EHDP with the precipitate of calcium phosphate, the distribution of EHDP-1-^{14}C between precipitate and filtrate was investigated. The results are shown in Table 1. When calcium and orthophosphate concentrations, temperature, time, and pH were held constant the ^{14}C-EHDP was always predominately associated with the precipitate (88.0 to 97.6%). This was true even when the concentration of the EHDP was 5×10^{-8} M (<0.02 ppm EHDP).

Table 1. *Distribution of EHDP-1-^{14}C between precipitate and filtrate (pH 7.40, 60 minutes reaction, $[CaCl_2] = 4.0 \times 10^{-3}$, $[NaH_2PO_4] = 4.0 \times 10^{-3}$)*

Concentration of EHDP-1-^{14}C (m/l)	Precipitate		Filtrate	
	cpm	% of total recovery	cpm	% of total recovery
1.2×10^{-4}	246,000	97.6	6,000	2.4
3.0×10^{-5}	74,500	95.1	3,800	4.9
1.0×10^{-6}	56,000	94.0	3,700	6.0
5.0×10^{-8}	4,520	88.0	600	12.0

The effect of EHDP and Cl_2MDP on the rate of precipitation of calcium phosphate is shown in Table 2. With similar conditions and times of precipitation, the concentration of calcium in the filtrate was about 33 and 8.4 times higher and phosphorus about 1.7 and 2.0 times higher in systems containing the two effective crystal growth inhibitors, EHDP and Cl_2MDP, respectively, than in the controls.

Fig. 3a—d. Structure modifications of calcium orthophosphate precipitates. a. Electron diffraction pattern of hydroxyapatite. The precipitate was formed from 1×10^{-2} M $CaCl_2$,

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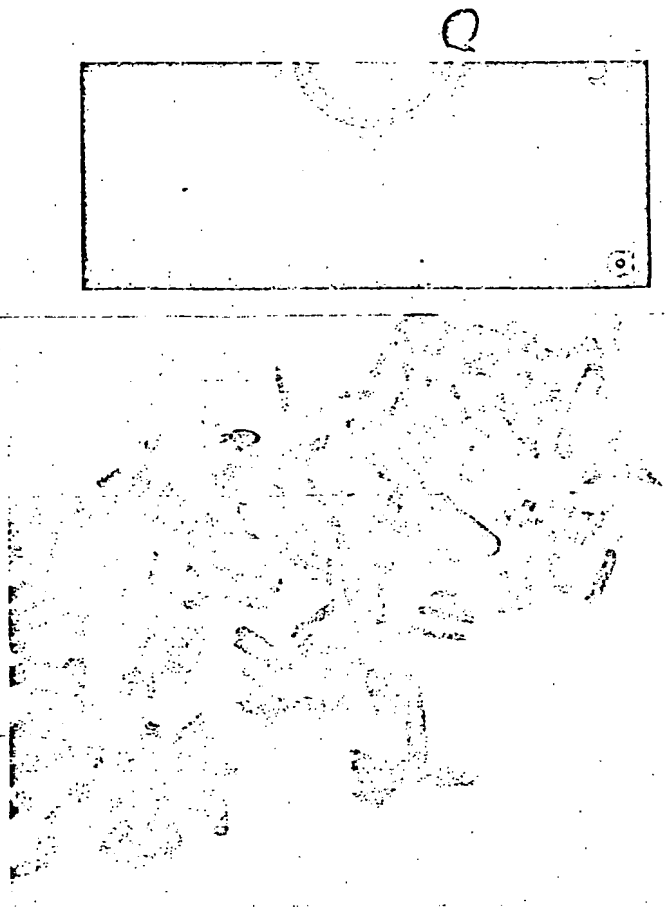

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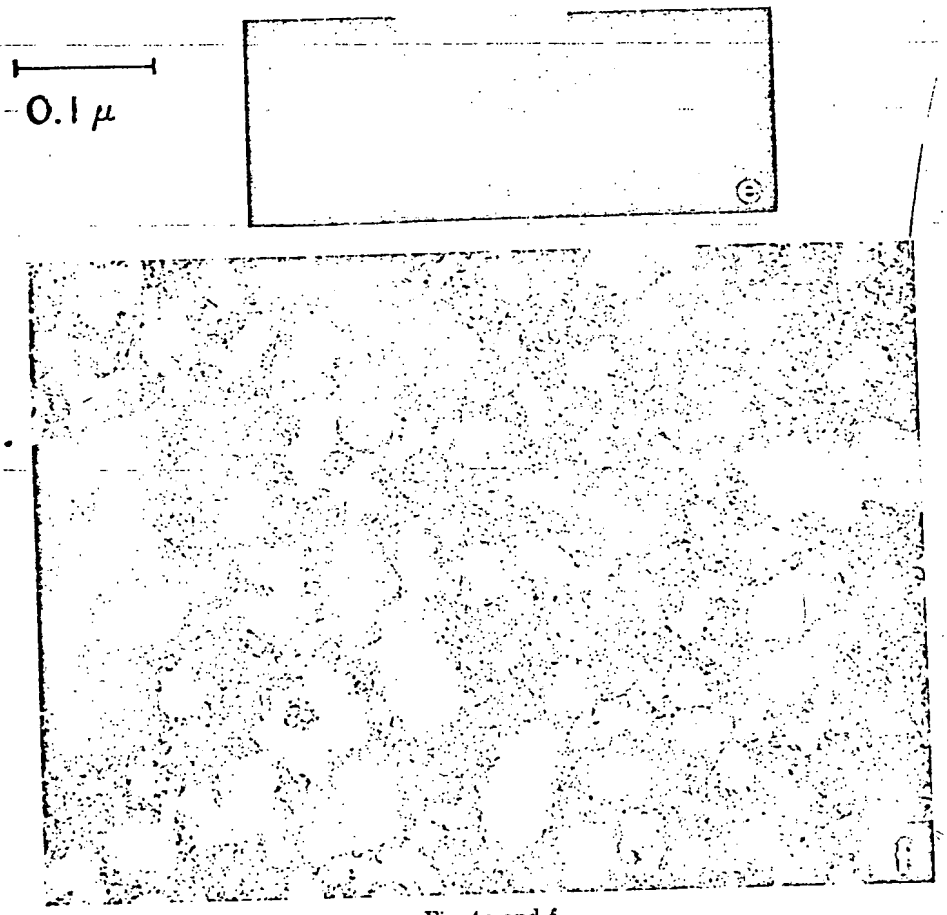

Fig. 4e and f

Fig. 4a—f. The effect of two different crystal modifiers on calcium orthophosphate precipitation. **a.** Spotty electron diffraction pattern of hydroxyapatite. The precipitate was formed from 1×10^{-2} M $CaCl_2$, 1×10^{-2} M NaH_2PO_4, pH 7.4, 48 hours reaction at 80°. b. Electron micrograph showing pronounced rectangular crystallites of hydroxyapatite. **c.** Spotty electron diffraction pattern of hydroxylapatite. The precipitate was formed from 1×10^{-2} M $CaCl_2$, 1×10^{-2} M NaH_2PO_4, 1×10^{-3} M $Na_4P_2O_7$, pH 7.4, 48 hours at 80°. d. Electron micrograph showing pronounced rectangular crystallites of hydroxyapatite not different from (b). **e.** Amorphous diffraction pattern. The precipitate was formed from 1×10^{-2} M $CaCl_2$, 1×10^{-2} M NaH_2PO_4, 1×10^{-3} M EHDP, pH 7.4, 48 hours at 80°. f. Electron micrograph of precipitate (e) showing non-crystalline, gel-like nature of precipitate even after 48 hours of reaction time. b, d and f ×180,000

The elevated calcium and phosphate concentrations probably result at least in part from the extremely small crystallite size of HA (amorphous) in the presence

The crossover experiment (Fig. 5) might suggest that EHDP prevents crystal growth of HA by physically covering the calcium orthophosphate precipitate by the formation of a calcium EHDP which then coprecipitates with the calcium orthophosphate and prevents crystal growth of the HA. From Table 1, however, it is evident that at EHDP concentrations far below those at which the calcium EHDP can precipitate (10^{-6} and 10^{-8} M EHDP) the EHDP-1-^{14}C was still entirely associated with the calcium orthophosphate precipitate. It is suggested, therefore, that the mechanism of the inhibition of crystal growth is one of strong chemisorption of EHDP on the microcrystallites of HA (possibly at screw dislocation sites) thus preventing the rapid addition of ions into the apatite lattice as occurs in the absence of the inhibitor. As judged by electron diffraction, addition of ions into the HA lattice in the presence of EHDP must not go beyond a few unit cells or the organized structures would have been detected. This limiting of the crystallite size probably also accounts for the colloidal suspension observed in the crossover experiment. The chemisorption properties of the EHDP also suggest the possible use of these polyphosphorates for therapy of osteoporotic problems in man and animals.

The author is grateful to R. L. Stewart for the synthesis of disodium-1-hydroxy-1,1-diphosphonate-1-^{14}C and to R. J. Neal for the electron micrographs and diffraction patterns, and to L. Flora and R. Hicks for technical assistance.

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The Influence of Pyrophosphate, Condensed Phosphates, Phosphonates and other Phosphate Compounds on the Dissolution of Hydroxyapatite *in vitro* and on Bone Resorption Induced by Parathyroid Hormone in Tissue Culture and in Thyroparathyroidectomised Rats

R. G. G. Russell, R. C. Mühlbauer, S. Bisaz, D. A. Williams, and H. Fleisch

Department of Pathophysiology, University of Berne, Switzerland,
Laboratory for Experimental Surgery, Davos, Switzerland,
Nuffield Orthopedic Centre, University of Oxford, England, and
Department of Statistics, University of Edinburgh, Scotland

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Earlier studies have shown that inorganic pyrophosphate (PP_i) inhibits the dissolution of hydroxyapatite crystals *in vitro* and it has been suggested that PP_i might be a physiological regulator of bone resorption. In this study PP_i and other phosphate compounds have been tested for their ability to inhibit bone resorption induced by parathyroid hormone in mouse calvaria and to inhibit the rise in plasma calcium induced by parathyroid hormone in thyroparathyroidectomised rats on a low calcium diet. Orthophosphate, pyrophosphate, polyphosphate and two polymeric phosphate inhibitors of phosphatases did not inhibit the resorption of calvaria or the rise in plasma calcium. In contrast, several phosphonates containing P-C-P bonds retarded the dissolution of hydroxyapatite crystals *in vitro*, and, at concentrations down to 1.6×10^{-6} M, inhibited bone resorption in tissue culture. Some diphosphonates also inhibited the rise in plasma calcium in thyroparathyroidectomised rats. One reason for the difference between the effects of compounds containing P-O-P and P-C-P bonds may be related to the greater resistance of the latter to chemical and enzymic hydrolysis. Phosphonates may provide a model for the effect of endogenous PP_i in bone, and might be of use in elucidating mechanisms of bone formation and resorption and in the therapy of diseases that involve increased resorption of bone.

Key words: Pyrophosphate—Orthophosphate—Phosphonates—Phosphates—Parathyroid—Bone.

Lors d'essais précédents, nous avons montré que le pyrophosphate minéral (PP_i) inhibe in vitro la dissolution de cristaux d'hydroxyapatite et nous avons suggéré que le PP_i pourrait être un régulateur physiologique de la résorption osseuse. Dans ce travail, nous avons cherché si le PP_i ainsi que d'autres produits phosphorés ont une action inhibitrice sur la résorption osseuse induite par l'hormone parathyroïdienne dans des calottes craniennes de souris et sur l'augmentation du calcium plasmatique induite par l'hormone parathyroïdienne chez des rats thyroparathyroïdectomisés maintenus en régime déficient en calcium. Ni l'orthophosphate, ni le pyrophosphate, ni les polyphosphates, ni deux phosphates polymérisés, inhibiteurs des phosphatases, n'ont inhibé la résorption des calottes craniennes, ni l'augmentation du calcium plasmatique. Par contre, certains phosphonates, contenant la liaison P-C-P, ont réduit la dissolution in vitro de cristaux d'hydroxyapatite et inhibé la résorption osseuse en culture de tissus à une concentration aussi faible que $1,6 \times 10^{-6}$ molaire. Certains diphosphonates ont inhibé également l'augmentation du calcium plasmatique chez des rats thyro-

For reprints: Dr. R. G. G. Russell, Nuffield Orthopedic Centre, University of Oxford, Oxford, Great Britain.

parathyroïdectomisés. La différence d'activité des produits contenant la liaison P-O-P ou P-C-P peut être attribuée à une plus grande résistance des derniers à une hydrolyse chimique ou enzymatique. Les phosphonates peuvent servir de modèle pour étudier l'action du PP_i endogène de l'os. Ils peuvent également être utiles pour éclaircir le mécanisme de la formation et de la destruction osseuse et pourraient jouer un rôle dans la thérapie des maladies présentant une résorption osseuse accrue.

Frühere Untersuchungen zeigten, daß anorganisches Pyrophosphat (PP_i) die Auflösung von Hydroxyapatit-Kristallen *in vitro* hemmt; es wurde vorgeschlagen, daß PP_i physiologisch die Knochenresorption regulieren könnte. Bei diesem Versuch wurden PP_i und andere Phosphatderivate auf ihre Eignung geprüft, die Knochenresorption zu hemmen, welche an Mäusecalvarien durch Parathormon hervorgerufen wurde; ebenso wurde abgeklärt, ob diese Substanzen in thyroparathyreoidektomierten, auf calciumarmer Diät gehaltenen Ratten in der Lage waren, den durch Parathormongaben verursachten Blutcalciumanstieg zu hemmen. Weder Orthophosphat, noch Pyrophosphat, Polyphosphat und zwei polymere Phosphatinhibitoren der Phosphatasen konnten die Resorption von Calvarien oder den Anstieg des Plasmacalciums hemmen. Dagegen verzögerten verschiedene Phosphonate mit P-C-P-Bindungen die Auflösung von Hydroxyapatit *in vitro* und hemmten dazu in so tiefen Konzentrationen wie $1{,}6 \times 10^{-6}$ M die Knochenresorption in der Gewebezucht. Einige Diphosphonate hemmten auch den Plasmacalciumanstieg bei thyroparathyreoidektomierten Ratten. Eine Ursache der unterschiedlichen Wirksamkeit von Substanzen, welche P-O-P- oder P-C-P-Bindungen enthalten, kann darin liegen, daß die letztgenannten einen größeren Widerstand gegenüber chemischer und enzymatischer Hydrolyse entgegenbringen. Phosphonate könnten als Modell für die Wirkung des endogenen PP_i im Knochen dienen und zudem zur Erläuterung der Mechanismen von Knochenbildung und -resorption nützlich sein; sie könnten auch zur Therapie jener Krankheiten, die eine erhöhte Knochenresorption zur Folge haben, herangezogen werden.

Inorganic pyrophosphate (PP_i) inhibits both the precipitation (Fleisch and Neuman, 1961; Fleisch *et al.*, 1966b) and dissolution (Fleisch *et al.*, 1966a) of hydroxyapatite crystals *in vitro*. Since PP_i is present in body fluids (Russell *et al.*, 1969) and in bone (Cartier, 1957; Perkins and Walker, 1958), it has been suggested that PP_i might regulate the rates of both entry and exit of calcium and phosphate in bone and that PP_i could thereby influence the rates of formation and destruction of mineralised tissues *in vivo*. The amount of PP_i present locally on the crystal surfaces in mineralised tissues would, in turn, govern the rates of entry and exit of the mineral, and the concentration of PP_i itself would be determined by the local activity of pyrophosphatases in bone (Fleisch and Russell, 1970). These pyrophosphatases would be separately controlled at sites of deposition and dissolution of mineral and might be influenced by hormones and other agents.

Although it has been shown that PP_i and longer-chain condensed phosphates can inhibit the deposition of calcium phosphate in chick embryo femora in tissue culture (Fleisch *et al.*, 1966c) and in various soft tissues *in vivo* (Gabbiani, 1966; Schibler and Fleisch, 1966; Schibler *et al.*, 1968), there have been no studies reported of the effect of PP_i and other condensed phosphates on the resorption of living bone.

The main purpose of the present study was to examine the effects of PP_i and related compounds on (1) the rate of dissolution of hydroxyapatite crystals *in vitro* and (2) on bone resorption in two systems:

a) induced by parathyroid hormone (PTH) in mouse calvaria in tissue culture,

and

b) induced by PTH in thyroparathyroidectomised (TPTX) rats. TPTX-animals on a low-calcium diet were used so that increases in plasma calcium might reasonably be attributed to bone resorption.

The phosphate compounds studied were:

1. inorganic pyrophosphate (PP_i) and a mixture of longer-chain condensed phosphates (polyphosphate);

2. orthophosphate (P_i); P_i was used as a control substance and because there were indications that it might itself inhibit bone resorption (Goldsmith and Ingbar, 1966; Raisz and Niemann, 1969);

3. polyphloretin phosphate and polyoestradiol phosphate; these compounds are polymeric phosphates which are inhibitors of phosphatases *in vitro* (Diczfalusy *et al.*, 1953) and therefore might have been expected to increase endogenous levels of PP_i *in vivo*;

4. various phosphonates (Table 1); the diphosphonates possess P-C-P bonds rather than the P-O-P bonds of PP_i and polyphosphates, and are resistant to spontaneous and enzymic hydrolysis. The effect of the diphosphonates on the precipitation of apatite *in vitro* and *in vivo* are similar to those of PP_i and the polyphosphates (Francis, 1969; Francis *et al.*, 1969; Fleisch *et al.*, 1970) and it has been suggested that such compounds might be used in human disease that involves abnormalities in calcium metabolism. Preliminary studies with three diphosphonates showed that they inhibit dissolution of apatite crystals *in vitro* and bone resorption in several systems (Fleisch *et al.*, 1969a; Fleisch *et al.*, 1969b). A total of ten phosphonates have now been studied and their effects are described in full in this paper.

Materials

Disodium orthophosphate and tetrasodium pyrophosphate were obtained from Merck A.G., Darmstadt, Germany. Graham Salt was a commercial preparation ("hexametaphosphate") of predominantly straight-chain polyphosphates (mean chain length of 25 units) obtained from J. A. Benkiser & Co., Ludwigshafen am Rhein, Germany. The phosphonates (see Table 1) were obtained through the courtesy of Dr. M. D. Francis of the Proctor and Gamble Company, Cincinatti, Ohio, USA. These particular phosphonates were chosen for the information they might yield regarding the chemical and steric nature of the structures necessary to inhibit bone resorption. Polyphloretin phosphate and polyoestradiol phosphate were a kind gift from Dr. O. Ferno of Leo & Co., Hälsingborg, Sweden.

For the tissue culture medium, all chemicals and reagents were of analytical grade wherever possible, generally from Merck & Co., Darmstadt, Germany; amino acids were from Fluka, Buchs, Switzerland; the vitamins were a generous gift from Hoffmann-La Roche; Streptomycin sulphate was from Pfizer and crystalline buffered Penicillin G from Ely Lilly Company.

Methods

Effects on Dissolution of Hydroxyapatite Crystals in vitro

Hydroxyapatite crystals (65 mg) from the same batch as we have used previously (Fleisch *et al.*, 1966b) were equilibrated with mechanical stirring for about 24 h at 37° in 200 ml solution of 0.155 M-KCl buffered at pH 7.0 with 0.01 M-barbital. After equilibration, 100 ml of the suspension was filtered and the wet crystals were returned to the remaining 100 ml suspension. PP_i or one of the phosphonates was dissolved in the 100 ml of filtrate, which was then added slowly back to the suspension with stirring over 2 h, so that the final amount of PP_i or phosphonate added (expressed as P) represented 5 % of the total phosphorus-P of the apatite present. One hour later, the entire batch of crystals was filtered (Millipore filters,

Table 1. *List of phosphonates studied*

Name of compound	Abbreviation	Formula (as acid)
n-pentanemonophosphonic acid	PMP	$CH_3(CH_2)_4—PO_3H_2$
ethane-1,2-diphosphonic acid	E-1,2-DP	$CH_2(PO_3H_2)—CH_2(PO_3H_2)$
methylenediphosphonic acid	MDP	$CH_2(PO_3H_2)_2$
methylenehydroxydiphosphonic acid	MHDP	$CH(OH)(PO_3H_2)_2$
dichloromethylenediphosphonic acid	Cl_2MDP	$Cl_2C(PO_3H_2)_2$
ethane-1-amino-1,1-diphosphonic acid	EADP	$CH_3C(NH_2)(PO_3H_2)_2$
ethane-1-hydroxy-1,1-diphosphonic acid	EHDP	$CH_3—C(OH)(PO_3H_2)_2$
ethane-1-hydroxy-1,1,2-triphosphonic acid	E-1-HTP	$CH_2(PO_3H_2)—C(OH)(PO_3H_2)—PO_3H_2$
propane-1,1,3,3-tetraphosphonic acid	PTeP	$CH(PO_3H_2)_2—CH_2—CH(PO_3H_2)_2$
polyester chain condensate of EHDP with acetic andydride	Condensate I	$CH_3—C(=O)—[O—C(CH_3)(PO_3H_2)—P(=O)(OH)]_{1.9}—C(CH_3)(PO_3H_2)_2$

0.45 μ pore size) and resuspended in 75 ml of 0.155 M-KCl containing 0.01 M-barbital at pH 7.0. Control crystals, treated in an identical manner but to which no PP_i or phosphonate had been added, were resuspended in a similar manner. The rate of dissolution of treated and control crystals was measured during the subsequent incubation at 37° with mechanical stirring. Aliquots were removed and filtered at 5, 30, 60 and 90 min and the concentration of Ca and P in the filtrates were determined by methods described elsewhere (Bisaz *et al.*, 1968).

Effects on Bone Resorption Induced by PTH in Mouse Calvaria in Tissue Culture

Medium. The composition of the basic medium we used is shown in Table 2. It is based on a modification (from the Strangeways Laboratory, Cambridge, England) of the BGJ medium described by Biggers *et al.* (1961). $CaCl_2$, $NaHCO_3$, phenol red and heat-inactivated

Table 2. *Composition of medium*

Column I	mg per 100 ml	Column II	mg per 100 ml	Column III	mg per 100 ml
L-arginine hydrochloride	17.5	Thiamin	0.4	KH_2PO_4	16.0
L-histidine hydrochloride	15.0	Riboflavin	0.02	$Na_2HPO_4 \cdot 2H_2O$	9.0
L-lysine	24.0	Pantothenate (calcium salt)	0.02	$NaHCO_3$	350.0
L-tyrosine	4.0	Biotin	0.02		
L-tryptophan	4.0	Folic acid	0.02		
L-phenylalanine	5.0	Choline chloride	5.0		
DL-methionine	5.0	α-tocopherol phosphate (sod. salt)	0.1	For other additions	
DL-serine	20.0	Nicotinamide	2.0	see text	
DL-threonine	7.5	Pyridoxal phosphate	0.02		
DL-leucine	5.0	Vitamin B_{12}	0.004		
DL-isoleucine	3.0	M-inositol	0.02		
DL-valine	6.5	p-aminobenzoic acid	0.2		
DL-aspartic acid	15.0	Ascorbic acid	5.0		
DL-alanine	25.0	Glucose	750.0		
L-proline	40.0	NaCl	530.0		
Glycine	80.0	KCl	40.0		
L-cysteine hydrochloride	9.0	$MgSO_4$	20.0		
L-glutamine	20.0	Sodium acetate	5.0		
Streptomycin sulphate	5.0				
Penicillin	10000 units				

horse serum was added to this medium at the concentrations described in the next paragraph. The actual procedure for preparing the medium was as follows:

Mixture I, which was prepared in bulk, consisted of those compounds listed in columns I and II of Table 2. Mixture II, also prepared in bulk, consisted of those compounds listed in column III of Table 2. When the medium was required, 1.874 g of I and 0.412 g of II were dissolved in 100 ml of distilled water. Then 0.2 ml of 1 M-$CaCl_2$, 1.76 ml of 0.6 M-$NaHCO_3$ and 0.22 ml of 1 % (w/v) phenol red were added and the volume made to 110 ml with water. The solution was then sterilised by Millipore filtration. Five millitres of heat-inactivated, sterile horse serum was then added to 95 ml of the sterile solution. The horse serum was obtained locally and was then heated at 57° for 30 min. It was then sterilised by Millipore filtration and stored frozen. After addition of horse serum, the solution was equilibrated with a 5 % CO_2, 20 % O_2, 75 % N_2 filtered gas mixture and was divided into two lots, one of which was used for rinsing the calvaria and for the control incubations and the other was taken for addition of parathyroid hormone and the various other compounds. The parathyroid hormone (PTH, Parathormone, Ely Lilly, 100 USP Units/ml) was first diluted (1:5) in sterile distilled water and was then added to the second batch of medium to provide a concentration of 0.2 USP Units/ml. It was essential to equilibrate the medium fully with 5 % CO_2 prior to addition of PTH in order to avoid precipitation. After gassing, this second batch of medium was then divided into aliquots for the addition of the various phosphate compounds. The phosphate compounds were prepared as sterile, concentrated, stock solutions (1 mg P/ml in water), adjusted to pH 7.4 with NaOH or HCl as appropriate and stored frozen.

Culture Technique. Calvaria were dissected using aseptic techniques from 2–4 day old NMRI mice from our laboratory stock. The calvaria were rinsed in medium and were then incubated individually at 37° in roller tubes (rotation speed 26 rev/h) each containing 2 ml of medium. The tubes were closed with silicone stoppers after the medium had been

gassed with a mixture of 5 % CO_2, 20 % O_2 and 75 % N_2. The medium was completely changed each day and the tube gassed again. After 4–5 days the calvaria were removed from the medium, fixed in 95 % ethanol for at least 2 h and stained.

Staining and Evaluation of Resorption. After fixation, the calvaria were washed in distilled water for 1 min and placed in a 5 % (w/v) solution of silver nitrate for 2 min. They were then rinsed in water and exposed to sunlight for 10 min. When blackening had occurred they were treated with 5 % (w/v) sodium thiosulphate for 1 min. After rinsing in water, they were dehydrated successively in 70, 95 and 100 % ethanol, cleared in xylol and mounted on a slide in "Eukitt" under a cover slip. With this technique areas of resorption showed up as clear regions against the darkly stained background of unresorbed bone. A quantitative estimate of the amount of resorption was made as follows. The image of each calvarium was projected onto a screen. A grid was placed over the image. The resorbed areas were then counted out as those places where clear regions could be seen under the points at which the wires of the grid intersected at right angles. The total area of the bone under the grid was determined by planimetry and recorded as the number of points of intersection under which any bone, resorbed or not, could be seen. The percentage of the total bone resorbed was then calculated as:

$$\frac{\text{number of points over clear areas} \times 100}{\text{total number of points over bone}}$$

The total number of points over the bone lay between 250–500. All the bones were examined by one observer. The 95 % confidence limits for the evaluation of % resorption in a single bone, when the measurements were made on different days, were within ± 2.5 % of the mean value.

Design and Analysis of the Experiment. The two series (Tables 3 and 4) each consisted of several experimental runs. In each run of the first series (Table 3) 3 calvaria were controls incubated with PTH, and the remaining 21 bones were allocated, usually in pairs, to the remaining treatments. Not all treatments were represented in each experimental run but each treatment was allocated to at least 10 calvaria over the series as a whole. In each experimental run of the second series (Table 4) 2 calvaria were controls without PTH, 6 were controls with PTH and 2 calvaria were allocated to each of the other eight treatments. The design in the second series was therefore a balanced randomised block design with replication of treatments within blocks.

Statistical analysis of the observations demonstrated that the mean resorption varied between experimental runs. For the first series in which the design was unbalanced the treatment means (Table 3) have been made comparable by adjustment for run differences. These adjustments were invariably small (Table 3). The estimates of the standard errors of the mean resorption for each treatment were obtained from the variance component for interaction between treatments and experimental runs.

Effects on Rise in Plasma Calcium Induced by PTH in Thyroparathyroidectomised Rats

Male Wistar rats, weighing 90–130 g, were thyroparathyroidectomised surgically and maintained on a diet low in calcium and phosphate (diet 199 OPC obtained from Nafag AG, Gossau, Switzerland, by analysis 1.66 g Ca/kg and 3.5 g P/kg). Their drinking water was supplemented with calcium gluconate (2 % w/v) immediately after operation until day 6 after operation. On the fourth day after thyroparathyroidectomy the animals were marked individually and placed in groups of 5 per cage. The various treatments with phosphate or phosphonate compounds and the control treatments were then allocated to animals at random and given daily over the next three days (day 5–7 after thyroparathyroidectomy). All compounds were dissolved in water and the pH adjusted to 7.4 with NaOH or HCl as appropriate, so that the daily dose could be given, subcutaneously or by mouth, in 0.2 ml/100 g body weight. A dose of 10 mg P/kg body weight for the phosphonates was chosen since preliminary studies on two phosphonates indicated that this dose gave a significant effect (Fleisch *et al.*, 1969a; Fleisch *et al.*, 1969b). From day 6 after thyroparathyroidectomy the animals continued to eat but were allowed to drink distilled water only. On the eight day after thyroparathyroidectomy no

Table 3. *Effect of phosphonates on bone resorption induced by PTH (0.2 U/ml) in mouse calvaria in tissue culture*

Treatment	Concentration of phosphonate (μg P/ml)	Number of calvaria	% of bone resorbed. Mean, not adjusted for differences between experimental runs (see text)	% of bone resorbed. Mean ± SE of mean, adjusted for differences between experimental runs (see text)
No addition	—	33	8.5	8.7 ± 0.9[b]
PTH alone	—	85	21.3	21.3 ± 0.7
PTH + PMP	1.0	13	24.0	24.0 ± 1.8
	10.0	9	17.6	18.1 ± 2.1
PTH + E-1,2-DP	1.0	14	21.4	21.1 ± 1.7
	10.0	12	19.4	19.6 ± 1.8
PTH + MDP	0.1	10	19.4	20.3 ± 2.0
	1.0	12	16.2	15.3 ± 1.9[b]
PTH + MHDP	0.1	13	16.3	16.9 ± 1.8[b]
	1.0	12	10.3	9.7 ± 1.9[b]
PTH + Cl_2MDP	0.1	13	10.1	10.9 ± 1.8[b]
	1.0	11	10.2	9.6 ± 1.9[b]
PTH + EADP	0.1	14	18.6	19.6 ± 1.7
	1.0	12	14.6	13.7 ± 1.9[b]
PTH + EHDP	0.1	14	13.7	14.6 ± 1.7[b]
	1.0	12	11.7	10.9 ± 1.9[b]
PTH + E-1-HTP	0.1	12	18.7	19.4 ± 1.8
	1.0	12	16.6	15.7 ± 1.9[b]
PTH + PTeP	1.0	13	24.7	23.7 ± 1.8
	10.0	10	17.5	17.8 ± 2.0[a]
PTH + Condensate I	0.1	12	16.8	17.6 ± 1.8[a]
	1.0	12	16.2	16.2 ± 1.9[b]

[a] Indicates reduction in mean % resorption compared with PTH alone is significant at the 5% level.

[b] Indicates reduction in mean % resorption compared with PTH alone is significant at the 1% level.

phosphate or phosphonate compounds were given. A blood sample was taken from the retro-orbital sinus and the animals were then injected once subcutaneously with 50 USP units bovine parathyroid extract (PTH, Para-thor-mone, Lilly, Indianapolis, USA) per 100 g body weight. Six hours after the injection of PTH, a second blood sample was taken. Calcium in plasma was determined by titration with EDTA using calcichrome (Bisaz *et al.*, 1968) as an indicator (for the results in Table 5) or (for the results in Table 6) by atomic absorption spectroscopy (Perkin Elmer Model 290 B). Two experiments were carried out as shown in Tables 5 and 6. In the first experiment (Table 5) ten different phosphonates were compared with each other. In the second experiment (Table 6) P_1, PP_1, polyphosphate, polyphloretin phosphate and polyoestradiol phosphate were compared with one of the most active phosphonates, namely Cl_2MDP. The differences between the mean plasma calcium values in the animals receiving PTH alone and those receiving no PTH or PTH plus phosphate or phosphonate compounds were examined statistically, using a pooled estimate of variance to assess the evidence for an effect of the phosphate or phosphonate compounds on the changes

Table 4. *Effect of orthophosphate, polyphosphate, polyphloretin phosphate and methylene diphosphonate on bone resorption induced by PTH (0.2 U/ml) in mouse calvaria in tissue culture*

Treatment	Concentration of compound added (μg P/ml)	Number of calvaria	% of bone resorbed mean ± SE of mean
No addition	—	18	6.8 ± 1.8[a]
Polyphosphate alone	32	17	14.3 ± 1.1[ab]
PTH alone	—	53	25.9 ± 1.8[b]
PTH + orthophosphate	16	18	23.8 ± 1.8[b]
	32	18	23.7 ± 1.8[b]
PTH + polyphosphate	16	18	28.5 ± 1.8[b]
	32	18	24.1 ± 1.8[b]
PTH + polyphloretin phosphate	10	18	24.3 ± 1.8[b]
PTH + MDP	0.1	18	19.1 ± 1.8[ab]
	1.0	18	15.4 ± 1.8[ab]

[a] Indicates reduction in mean % resorption compared with PTH alone is significant at the 1% level.

[b] Indicates enhancement of mean % resorption compared with control (no addition) is significant at the 1% level.

induced by PTH. The effects of the phosphates and phosphonates on the initial plasma calcium measured before PTH was given were also examined using a pooled estimate of variance.

Results

Dissolution of Hydroxyapatite Crystals

The effects of the various phosphonates on the dissolution of hydroxyapatite crystals *in vitro* are shown in Fig. 1. Under the particular conditions of this experiment PP_i inhibited dissolution to the greatest extent. MDP, EADP, EHDP, MHDP, PTeP and E-1-HTP also caused a marked inhibition of dissolution. Cl_2MDP and E-1, 2-DP inhibited to a smaller extent, whereas PMP and Condensate I had virtually no effect.

Resorption of Calvaria in Tissue Culture

The effects of the phosphonates on the resorption of mouse calvaria in tissue culture are shown in Table 3. Parathyroid hormone added at 0.2 U/ml induced an easily detectable resorption in such bones in culture. Four compounds, namely MHDP, Cl_2MDP, EHDP and Condensate I, produced a significant inhibition of this resorption when added at 0.1 μg P/ml. These compounds were also inhibitory at 1.0 μg P/ml and at this concentration three other compounds, MDP, EADP and E-1-HTP, also had a significant effect. PTeP produced a significant inhibition at 10 μg P/ml, whereas compounds PMP and E-1, 2-DP had no inhibitory effect at this concentration. Table 4 shows that P_i, polyphosphate and polyphloretin phosphate did not inhibit the bone resorption induced by PTH, even when they were added at concentrations up to 32 μg P/ml. In this series of experiments MDP at 1.0 μg P/ml inhibited bone resorption as it did in the first series

Fig. 1. symbol *in vitro* Ca × P contain standa

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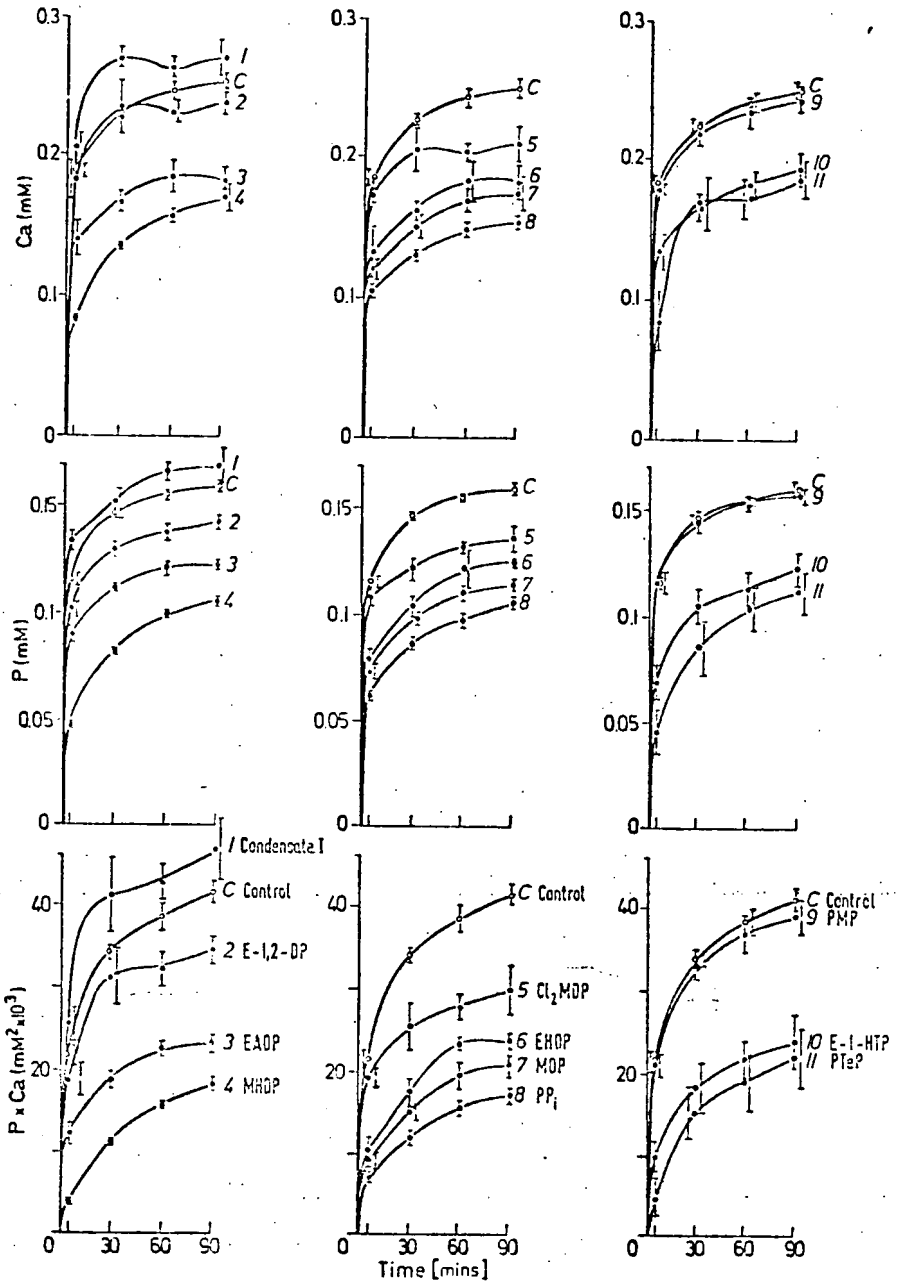

Fig. 1. Effect of pretreating hydroxyapatite crystals with phosphonates (see Table 1 for symbols) or with pyrophosphate (PP_i) on the subsequent rate of dissolution of the crystals *in vitro*. The figure shows the concentrations of calcium (*Ca*) and phosphate (*P*), and the Ca × P product, in solution at various times after adding treated crystals to a buffer initially containing no calcium or phosphate (see text for details). Each point represents the mean ± standard error of mean for at least 4 separate experiments (9 experiments in the case of control non-treated crystals—denoted C)

Table 5. *Effect of various phosphonates on plasma calcium (mg/100 ml) measured before and after injection of parathyroid hormone (PTH, 500 USP Units/kg body weight) into thyroparathyroidectomised rats. Phosphonates were given daily at 10 mg P/kg body weight for three days up to but not including day of injection of PTH*

Treatment		Initial plasma calcium before injection of PTH			Change in plasma calcium 6 h after PTH		
		Number of animals	Mean	± SE of mean	Number of animals	Mean	± SE of mean
No treatment		18	6.5	± 0.2	18	− 0.5[b]	± 0.3
PTH alone		52			52	+ 1.8	± 0.2
PTH + PMP	s.c.	11	6.1	± 0.5	11	+ 2.0	± 0.4
PTH + PMP	p.o.	10	6.1	± 0.5	10	+ 2.3	± 0.4
PTH + E-1,2-DP	s.c.	10	5.9	± 0.5	10	+ 1.5	± 0.4
PTH + E-1,2-DP	p.o.	9	8.3[b]	± 0.5	8	+ 1.1	± 0.5
PTH + MDP	s.c.	7	5.6	± 0.6	7	0.0[b]	± 0.5
PTH + MDP	p.o.	11	6.6	± 0.5	10	+ 1.8	± 0.4
PTH + MHDP	s.c.	3	4.7	± 0.9	3	+ 0.1[a]	± 0.7
PTH + MHDP	p.o.	11	6.1	± 0.5	11	+ 1.8	± 0.4
PTH + Cl_2MDP	s.c.	12	5.6	± 0.5	12	+ 0.1[b]	± 0.4
PTH + Cl_2MDP	p.o.	10	5.7	± 0.5	10	+ 0.8[a]	± 0.4
PTH + EADP	s.c.	10	7.6[a]	± 0.5	10	− 0.5[b]	± 0.4
PTH + EADP	p.o.	9	6.9	± 0.5	8	+ 1.8	± 0.5
PTH + EHDP	s.c.	12	8.4[b]	± 0.5	12	+ 0.8[a]	± 0.4
PTH + EHDP	p.o.	11	6.5	± 0.5	11	+ 1.4	± 0.4
PTH + E-1-HTP	s.c.	8	7.8[a]	± 0.6	8	+ 2.0	± 0.5
PTH + E-1-HTP	p.o.	12	6.2	± 0.5	12	+ 1.6	± 0.4
PTH + PTeP	s.c.	9	7.2	± 0.5	9	+ 1.9	± 0.4
PTH + PTeP	p.o.	7	5.8	± 0.6	6	+ 1.2	± 0.5
PTH + Condensate I	s.c.	11	8.6[b]	± 0.5	11	+ 1.9	± 0.4
PTH + Condensate I	p.o.	11	6.7	± 0.5	10	+ 2.1	± 0.4

[a] Indicates that mean is significantly different from control mean at 5% level.
[b] Indicates that mean is significantly different from control mean at 1% level.
Control = no treatment + PTH alone for initial plasma calcium. Control = PTH alone for change in plasma calcium.

(see Table 3), but, in addition, the 0.1 µg P/ml dose was also effective. Addition of polyphosphate at 32 µg P/ml in the absence of PTH caused a significant enhancement of bone resorption as measured by this technique.

The method used to assess bone resorption in tissue culture proved both sensitive and reliable. There was a close similarity between the quantitative response of the calvaria to PTH in the two series of experiments shown in Tables 3 and 4. In preliminary studies in collaboration with Drs. J. Reynolds and C. Minkin using calvaria from mice injected with ^{45}Ca, both Cl_2MDP and EHDP slow the release of ^{45}Ca and ^{45}Ca from such bones in culture.

Effects on Plasma Calcium in Thyroparathyroidectomised Rats

1. Effect on the Increase in Plasma Calcium Induced by PTH. In both experiments PTH given at 50 USP units/100 g body weight induced a significant rise in mean plasma calcium over 6 hours (Tables 5 and 6). This rise in plasma calcium

Table 6. *Effect of orthophosphate, pyrophosphate and various polymeric phosphates on plasma calcium (mg/100 ml), before and after injection of parathyroid hormone (PTH, 500 USP Units/kg body weight) into thyroparathyroidectomised rats. All compounds given daily over three days up to but not including day of injection of PTH*

Treatment	Dose mg P/kg given s.c.	Number of animals	Initial plasma calcium before injection of PTH		Change in plasma calcium 6 h after PTH	
			Mean	± SE of mean	Mean	± SE of mean
No treatment	—	13	6.3	±0.1	−1.1[b]	±0.3
PTH alone	—	41			+2.5	±0.2
PTH + orthophosphate	10	12	6.3	±0.3	+2.3	±0.3
PTH + orthophosphate	30	15	6.1	±0.3	+2.9	±0.3
PTH + pyrophosphate	10	14	6.7	±0.3	+3.0	±0.3
PTH + pyrophosphate	30	12	6.6	±0.3	+2.4	±0.3
PTH + polyphosphate	10	13	7.0[a]	±0.3	+2.5	±0.3
PTH + polyphosphate	30	15	7.1[b]	±0.3	+2.3	±0.3
PTH + polyphloretin phosphate	30	14	6.2	±0.3	+3.0	±0.3
PTH + polyestradiol phosphate	30	12	5.8	±0.3	+2.1	±0.3
PTH + Cl_2MDP	10	11	5.3[b]	±0.3	0.0[b]	±0.3

[a] Indicates that mean is significantly different from control mean at 5% level.
[b] Indicates that mean is significantly different from control mean at 1% level.
Control = no treatment + PTH alone for initial plasma calcium. Control = PTH alone for change in plasma calcium.

was partially or completely prevented by 5 of the phosphonates, namely MDP, MHDP, Cl_2MDP, EADP and EHDP, all given subcutaneously. Cl_2MDP was also effective by mouth (Table 5). The other phosphate and phosphonate compounds had no significant effects (Tables 5 and 6).

2. Effects on Initial Plasma Calcium before Injection of PTH. Some of the phosphate compounds had significant effects on the plasma calcium measured before injection of PTH. These values were obtained about 20 hours after the last injection of phosphate or phosphonate compounds. There was evidence for an increase in mean initial calcium compared with control (pooled values from control and PTH only) with the polyphosphate at both doses. Significant increases from control also occurred with five of the phosphonates, namely E-1,2-DP p.o., EADP s.c., EHDP s.c., E-1-HTP s.c. and Condensate I s.c. Cl_2MDP caused a depression in mean initial calcium in both experiments (Tables 5 and 6) but this change was statistically significant only in the second experiment.

Discussion

The main results of the various experiments are summarised in simple form in Table 7. Several phosphonates retarded the dissolution of apatite crystals *in vitro* as did PP_i. Some of the phosphonates also inhibited bone resorption in tissue culture, even when added at concentrations as low as 1.6×10^{-6} M. Under similar experimental conditions, P_i, polyphosphate and polyphloretin phosphate did not inhibit bone resorption, even when added at much higher concentrations.

Table 7. *Summary of effects of various phosphate compounds on the dissolution of hydroxyapatite crystals in vitro, on bone resorption in tissue culture and on plasma calcium in thyroparathyroidectomised rats. For experimental conditions – see text*

Compound	Inhibition of dissolution of hydroxyapatite *in vitro*	Inhibition of PTH-induced resorption of mouse calvaria in culture	Effects in thyroparathyroidectomised rats	
			Inhibition of rise in plasma calcium induced by PTH	Effect on plasma calcium in absence of PTH + = ↑; − = ↓
PP_i	+++	*	0	0
Polyphosphate	*	0	0	+
P_i	*	0	0	0
Polyphloretin phosphate	*	0	0	0
Polyoestradiol phosphate	*	*	0	0
PMP	0	0	0	0
E-1,2-DP	+	0	0	+
MDP	++	++(+)	++	0
MHDP	++	+++	+	0
Cl_2MDP	+	+++	+++	−
EADP	++	++	++	+
EHDP	++	+++	+	+
E-1-HTP	++	++	0	+
PTeP	++	+	0	0
Condensate I	0	+++	0	+

* Denotes experiment not done.

In the experiments on thyroparathyroidectomised rats, P_i, PP_i and polyphosphate, even at relatively high doses, had no detectable effect on the rise in plasma calcium induced by PTH. Phosphatase inhibitors (polyphloretin phosphate and polyoestradiol phosphate) were equally ineffective. In contrast, five of the diphosphonates tested were able to partially or completely prevent the rise in plasma calcium. It is not certain that the effect on plasma calcium can be explained solely in terms of changes in bone resorption, since we did not measure other changes, such as the renal handling of calcium or calcium content of soft tissues.

The correlation between the effectiveness of particular phosphonates in thyroparathyroidectomised rats and in tissue culture was good, but the correlation between the effect of any single phosphonate on bone resorption and its effect on the inhibition of crystal dissolution *in vitro* was not clear cut. For instance, Cl_2MDP was an extremely effective inhibitor in tissue culture and in rats but was among the least effective on crystal dissolution. Similarly, Condensate I had some effect in tissue culture but none on crystal dissolution. The effect of compounds in the rats could be influenced by their rate of mobilisation from the site of injection or their rate of absorption through the gastrointestinal tract. The molecular size of the compounds tested is probably also a

factor that determines biological activity in the rats, since the larger phosphonates, e.g. E-1-HTP, PTeP and Condensate I, did not prevent the effects of PTH in the TPTX rats. Failure to gain access to the site of action on account of molecular size may explain why the phosphatase inhibitors had no effect in any of the systems. The failure of the condensed phosphates to inhibit bone resorption may be related to the ease with which they are destroyed by enzymes. It is otherwise difficult to understand why they should fail to act under circumstances where the closely related diphosphonates are effective. PP_i, for instance, is very rapidly destroyed when given to living animals (Jung *et al.*, 1970), whereas the phosphonates have proved resistant to all enzymes so far tested. Phosphonates containing single C-P or P-C-C-P bonds (PMP and E-1,2-DP, respectively) were inactive in all systems. This strengthens the view that the P-C-P structure is required for the effects on crystal dissolution as well as in living systems. Indeed, in the light of all available evidence, the most plausible explanation of the effects of the phosphonates on the calvaria and in the thyroparathyroidectomised rats is that they retard the dissolution of bone crystals and thereby prevent parathyroid hormone from exerting its full effect. However, other possible mechanisms of action cannot be excluded, such as effects on cell metabolism, enzymes, or cellular ion transport systems.

The lack of effect of orthophosphate (P_i) on bone resorption in our experiments is interesting in view of the current therapeutic use of P_i to reduce hypercalcaemia (Goldsmith and Ingbar, 1966) and to accelerate fracture healing (Goldsmith *et al.*, 1967) in man. Raisz and Niemann (1969) found that P_i does inhibit bone resorption in tissue culture, but they used a larger range of concentrations of P_i and a different experimental system.

It is also interesting that some of the compounds which were unable to inhibit the effect of PTH on plasma calcium in TPTX rats did, however, cause changes in the initial plasma calcium measured before PTH was given. Thus, E-1,2-DP, EHDP, EADP, E-1-HTP, Condensate I and the polyphosphate all increased the initial plasma calcium. In contrast Cl_2MDP depressed the initial plasma calcium in the TPTX rats. The reason for these effects is unknown and further studies will be necessary in order to define the effects of these compounds on calcium turnover in bone, kidney, intestine and other tissues.

The observation that stable compounds related to the condensed phosphates inhibit bone resorption strengthens the hypothesis that endogenous PP_i in bone may be important in the physiological regulation of bone resorption. The diphosphonates may prove valuable tools for elucidating mechanisms of bone formation and resorption and may provide a convenient model for studying the effects of PP_i in bone. These observations may also provide a basis for the rational therapy of human diseases in which calcium metabolism is disturbed.

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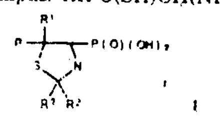
(1)

...)acrylate with $Cl(CH_2)_3SiR^1(OR^2)_2$ in the presence of a ...phonium salt, e.g. Bu_4PCl or Ph_3PEtBr. Thus, 0.1 mol K ...acrylate was heated with 0.1 mol $Cl(CH_2)_3Si(OMe)_3$, 0.002 mol Bu_4PCl, and 0.1 g *N,N'*-dinaphthyl-*p*-phenylenediamine in ...Me at 115° 3 h to give 94% I (R = R^2 = Me, R^1 = OMe), vs. ...% with Bu_4NCl instead of Bu_4PCl. I. Matsumoto

...52497u **1,3-Dimethyl-1,3-divinyl-1,3-di(N-ethylacet=...)disiloxane.** Shin-Etsu Chemical Industry Co., Ltd. ...Kokai Tokkyo Koho JP 81,104,888** (Cl. C07F7/10), 20 ...81, Appl 80/7,636, 25 Jan 1980; 3 pp. Disiloxane ...SiMe(CH:CH$_2$)]$_2$O (I), useful for chain-elongation of ...siloxanes, was prepd. by treating $[ClSiMe(CH{:}CH_2)]_2O$ (II) ... AcNHEt. Thus, 1.3 mol AcNHEt was added dropwise to ... mol II and 3.0 mol Et_3N in PhMe at 25–55° 30 min and the ...mixt. stirred at 60° 2 h to give 75% I. I. Matsumoto

...52498v **Antiinflammatory aminoalkanephosphinic and phosphonic acids.** Andrews, Kenneth John Maynard (Hoffmann-La Roche, F., und Co. A.-G.) **Eur. Pat. Appl. EP 33,919** (Cl. C07F9/48), 19 Aug 1981, GB Appl. 80/3,420, 01 Feb 1980; ... pp. Five title compds. $RR^1C(SH)CH(NH_2)P(O)(OH)X$ (R =



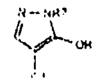
I

...R^1 = H, alkyl, X = H, OH, alkyl, aryl) were prepd. by ...lytic ring cleavage of I (R, R^1, X = same as above, R^2 = ...). Thus, 1.5 g DL-(2,2,5,5-tetramethyl-4-thiazolidinyl)phosphinic ... was hydrolyzed to give 0.85 g DL-(1-amino-2-mercapto-2-=...propyl)phosphinic acid, which was resolved. Similarly ...d were DL-$Me_2C(SH)CH(NH_2)P(O)(OH)X$ (X = OH, Me, ...)

...52499w ***N*-(Phenylphosphonomethyl)aminoacetonitrile.** ... Chemical Co., Ltd. **Jpn. Kokai Tokkyo Koho JP** ...,865 (Cl. C07F9/40), 20 Aug 1981, Appl. 80/7,358, 23 ... 3 pp. Herbicidal title compd., $HO_2P(OPh)CH_2NHCH_2CN$ ... prepd. by hydrolysis of $[(PhO)_2P(O)CH_2N(CH_2CN)]_2CH_2$ ... Thus, 0.1 mol $(PhO)_2POH$, heated with 0.05 mol 1,3,5-tris=...methylhexahydro-s-triazine and 3 mL 47% $BF_3.Et_2O$ at ... h gave 69.8% II, which (0.05 mol) was refluxed with 1.8 ... MeCN 4 h to give 53.4% I. I. Matsumoto

...52500q **Vinylphosphonic acid derivatives.** Kleiner, ...(Hoechst A.-G.) **Ger. Offen. DE 3,014,737** (Cl. C07F9/40), 22 Oct 1981, Appl. 17 Apr 1980; 10 pp. ...hydrohalogenation of $ClCH_2CH_2P(O)(OMe)_2$ at 195° 6 h gave ...% $H_2C{:}CHP(O)(OMe)_2$, 32% $H_2C{:}CHP(O)(OMe)(OH)$, 10% $H_2C{:}CHP(O)(OH)_2$, 1% di-Me vinylpyrophosphonate, 10% Me vinylpyrophosphonate, and 12% vinylpyrophosphonic acid. $ClCH_2CH_2P(O)(OEt)_2$ reacted similarly.

96: 52501r **Pyrazol-5-yl-(thio)(thiol)phosphoric (phosphonic) ... ester and their use for controlling pests.** Loeffler, Hans ...; Hutmacher, Hans Martin; Adolphi, Heinrich (BASF ...) **Ger. Offen. DE 3,013,291** (Cl. C07F9/65), 15 Oct ..., Appl. 05 Apr 1980; 29 pp. Approx. 50 insecticidal title



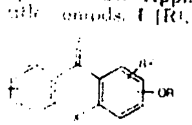
I, R=OP(X)(OR')R²
II, R=H

compds. I, X = S, O, R^1 = C_{1-4} alkyl, R^2 = Me, Et, Ph, amino, ... $C_{...}$ alkoxy, C_{1-6} alkylthio; R^3 = H, C_{1-6} alkyl, C_{3-6} ...alkyl, Ph; R^4 = C_{1-4} alkyl] were prepd. by esterification of ... $ZP(X)(OR^1)R^2$ (Z = halo). Thus, 5.6 g 1,4-dimethyl-5-=...hydroxypyrazole was esterified with 7.5 g $ClP(S)(OEt)_2$ to give ...I (R^1 = Et, R^2 = EtO, R^3 = R^4 = Me, X = S).

...52502s **Diphosphonic acid derivative.** Buxade Vinas, ... (Laboratorios Vinas S. A.) **Span. ES 493,010** (Cl. ...), 01 Jun 1981, Appl. 01 Jul 1980; 5 pp. HOCMe[P=...] (I) was obtained in 26.6% yield from HOAc and ... The LD_{50} for the pharmacol. active I in rats was 235

...52503t ...-Amino-1-hydroxyalkylidene-1,1-bisphosphonic ... Blum, Helmut; Worms, Karl Heinz (Henkel K.-G.a.A.) ...Offen. DE 3,016,289** (Cl. C07F9/38), 29 Oct 1981, Appl. ... Apr 1980; 11 pp. Phosphonylation of $H_2N(CH_2)_nCO_2H$ gave ...% $H_2N(CH_2)_nC[P(O)(OH)_2]_2OH$ (*n* = 3, 5), useful as ...chelating agents.

...52504u **Phosphorus esters, insecticide composition and ...od for insect control.** Traxler, James T. (Velsicol ...) **Braz. Pedido PI BR 81 01,100** (Cl. ...), ...8 Sep 1981, US Appl. 127,007, 04 Mar 1980; 32 ... title compds. I [R^1, R^2 = halo, haloalkyl, NO_2,



I, R=P(Z)R³R⁴
II R=H

alkylsulfonyl, alkylsulfinyl, cyano; X, Y, Z = O, S; R^3 = alkoxy, substituted PhO; R^4 = alkyl, alkoxy, alkylthio, amino, substituted Ph] were prepd. by esterifying II with $ClP(Z)R^3R^4$. Thus, 0.004... mol 2-hydroxyxanthen-9-one and 0.0049 mol $ClP(S)(OEt)_2$ gave I (R^1 = R^2 = H, R^3 = R^4 = OEt, X = Y = O, Z = S) (II). At K... ppm II gave 100% kill of mosquitoes.

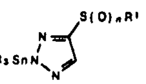
96: 52505v **Triarylphosphonium sulfobetaines.** Lapin, A. A.; Pravdin, V. G.; Bochkarev, Yu. A.; Romanov, G. V.; Pudovik, A. N. (All-Union Scientific-Research and Design Institute of Surfactants; Arbuzov, A. E., Institute of Organic and Physical Chemistry) **U.S.S.R. SU 859,371** (Cl. C07F9/54), ... Aug 1981, Appl. 2,868,902, 11 Jan 1980. From *Otkrytiya, Izobret., Prom. Obraztsy, Tovarnye Znaki* 1981, (32), 115-16. Title substances $R_3P^+SO_3^-$ (R = Ph or Me substituted Ph) were prepd. with an increased yield by treating triarylphosphines with a gas-air mixt. contg. 7% by vol. SO_3 in an inert org. solvent at 0–20°.

96: 52506w **2-Chloroethanephosphonic acid.** Handke, Rainer; Jahn, Heinz; Kern, Edgar; Kluger, Karl Heinz; Kryslak, Paul; Kochmann, Werner; Steinke, Walter; Gloede, Joerg; Gross, Hans (VEB Chemiekombinat Bitterfeld) **Ger. (East) DD 149,860** (Cl. C07F9/38), 05 Aug 1981, Appl. 204,325, 22 Mar 1978; 9 pp. The title compd. was prepd. in 39–87% yield by hydrolysis of $ClCH_2CH_2P(O)(OCH_2CH_2Cl)_2$ with HCl at 155-80°.

96: 52507x **Phosphoenolpyruvic acid.** Chisso Corp. **Jpn. Kokai Tokkyo Koho JP 81,113,789** (Cl. C07F9/09), 07 Sep 1981, Appl. 80/2,469, 12 Jan 1980; 5 pp. A mixt. of $MeCOCO_2H$ 1, Me_3SiCl 2.2, Et_3N 2.2, and 4-dimethylaminopyridine 0.017 mol. in C_6H_6 was refluxed 2 h to give 91% $Me_3SiOC({:}CH_2)CO_2SiMe_3$, which was reacted with 1 mol Br in CH_2Cl_2 ... min at −78° and then with 0.9 mol. $Me_3SiOP(OMe)_2$ overnight at 0° to give 90% $(Me_3SiO)_2P(O)OC({:}CH_2)CO_2SiMe_3$ (I), which was treated with NaOEt in Et_2O at 3° to give 98% $(NaO)_2P(O)OC({:}CH_2)CO_2Na$ (II). K. Sempuku

96: 52508y **Phosphazenic esters with fireproofing properties.** Schulz, Paul; Vilceanu, Radu (Centrul de Chimie, Timisoara) **Rom. RO 67,311** (Cl. C07F9/24), 29 Aug 1979, Appl. 85,9..., 05 May 1976; 2 pp. The title compds., $[-PR^1R^2{:}N-]_n$ (R^1, R^2 = C_{2-8} alkoxy, *n* = 3–10), were prepd. by cyclizing PCl_5 with NH_4Cl followed by treatment with R^1OH and R^2OH.

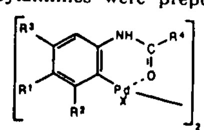
96: 52509z **Trialkyl-(1,2,3-triazolyl)-tin compounds and a biocide containing these compounds.** Krueger, Hans Rudolf; Schroeer, Ulrich; Baumert, Dietrich; Joppien, Hartmut (Schering A.-G.) **Ger. Offen. DE 2,936,951** (Cl. C07F7/22), 2... Apr 1981, Appl. 10 Sep 1979; 44 pp. Stannylation of



I

1,2,3-triazoles with distannoxanes gave 30 stannyltriazoles I (R = cyclohexyl, Bu; R^1 = C_{1-10} alkyl, C_{3-8} alkenyl, alkynyl, C_{1-4} substituted alkyl; *n* = 0–2). Thus, 0.015 mol $(R_3Sn)_2O$ (R = cyclohexyl) was refluxed 3 h with 4-(methylthio)-1,2,3-triazole in 100 mL acetone to give 83% I (R = cyclohexyl, R^1 = Me, *n* = 0)(II).

96: 52510t **2-Vinyl-N-acylanilines.** Kawaken Fine Chemicals Co., Ltd. **Jpn. Kokai Tokkyo Koho JP 81 86,141** (Cl. C07C103/34), 13 Jul 1981, Appl. 79/162,255, 14 Dec 1979; 4 pp. 2-Vinyl-*N*-acylanilines were prepd. by treatment of Pd



I

complexes I (R^1, R^2, R^3 = H, Cl, C_{1-4} alkyl, or alkoxy, acyl, alkoxycarbonyl, R^4 = C_{1-4} alkyl, X = C_{2-4} fatty acid residue) with $CH_2{:}CZY$ (Z = H, C_{1-8} alkyl, aryl, cyano, acyl, acyloxy, Y = Z or alkoxycarbonyl). Thus, *N*-acetylanilinoacetoxycyclopalladium dimer (19, 0.0016 mol) was refluxed with styrene (0.312 g, 0.003 mol), and Et_3N (1.0 g, 0.01 mol) in PhMe (10 mL) to give, after conventional workup 59.6% 2-acetamidostilbene.

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For papers of related interest see also Section:

20 51400v Structural data bank and its use.
22 51639e Role of dioxygen as an activator in olefin metathesis.
24 51851t Conjugate addition of trialkylaluminum and lithium benzenethiolate with enol phosphate of 1,3-dicarbonyl compounds.
51856y The chemistry of 4,4-dialkylcyclohex-2-enylidene... Phenylcarbenes by phenylcarbene rearrangement.
28 52210v Isoxazoles as β-diketone synthons. Selective ... formation on 3,5-dialkylisoxazoles.
52243h 1,3,2-Thiazaphospholidin-2-ones derived from ephedrine. Preparation and stereochemistry of ring-opening reactions.
52277x Silicon derivatives of medicinal agents: derivatives of p-aminosalicylic, salicylic and benzoic acids.
30 52519c Thermal reactions of B-alkyl-9-borabicyclo[3.3.1]nonanes

(9-BBN). E...
B-pinanyl-9-...
31 52568t St...
reserpine.
35 35775r Al...
complexes of...
intermediate.
35780p Zi...
35813b Sy...
35824f Sy...
rinated chain...
39 Industrial...
41 40455r Bi...
role of meth...
methylation i...
43 41250g A...
siloxanes.
41286y Ul...
aqueous copp...
46 41394g In...
borane cage.
alkynes.
75 44143k Sy...
p-quinodimet...
[Co^{III}Co^{III}] tri...
44144m C...
...pentadienyl
44145n Cr...
44149s Cr...

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96: 52511u R...
sesquiterpene...
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spirovetivanes...
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96: 52512v Th...
hydrocarbons.
using fused sa...
Coll. Eng., Kin...
Kenkyu Hokoku...
...-pinene, 2(10)-...
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(*E*,6*E*/4*E*,6*Z*)-...
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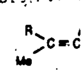
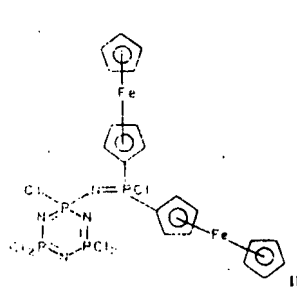


...(propenyl)cyclop...
... R = Ph, Me, ...
..., which was ob...
... = CH_2CO_2Me...
96: 52514x Pae...
...peony roots. S...
...; Kimura...
...chi, Hiroshi;...
.... Toyama M...
Tetrahedron Lett...



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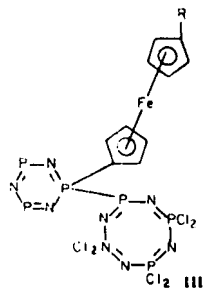
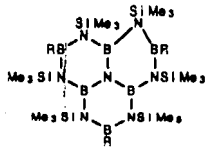


III

example of a trimer–tetramer bi(cyclophosphazene).

100: 175058k **Chemistry of boron. Part 144. Synthesis and structure of a B_4N_7-phenalene.** Gasparis-Ebeling, Theo; Noeth, Heinrich (Inst. Anorg. Chem., Univ. Muenchen, D-8000 Munich, 2 Fed. Rep. Ger.). *Angew. Chem.* 1984, 96(4), 301 (Ger).



I

Cyclization of $Me_3SiN(SnMe_3)_2$ with $B(SMe)_3$ gave 86% I (R = SMe) (II) which was treated with BCl_3 to give I (R = Cl). The crystal structure of II was detd.

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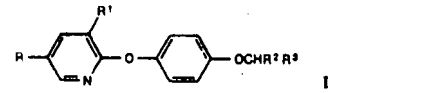
100: 175059m **Simultaneous manufacture of trialkylboron and alkyldihaloaluminum compounds.** Synoradzki, Ludwik; Boleslawski, Marek; Pasynkiewicz, Stanislaw; Jaworski, Krzysztof; Zawadzki, Mieczyslaw; Ratajczak, Lucjan (Politechnika Warszawska; Instytut Ciezkiej Syntezy Organicznej "Blachownia") **Pol. PL 120,074** (Cl. C07F5/06), 25 Jul 1983, Appl. 215,916, 28 May 1979; 2 pp. R_3B and $RAlX_2$ were prepd. simultaneously by reaction of B_2O_3 with sesquialkylchloroaluminum. Thus, a mixt. of 500 g $Me_3Al_2Cl_3$ contg. 60% $MeAlCl_2$ and 25 g B_2O_3 was stirred 10 h at 170° to give 38.1 g Me_3B and 298 g $MeAlCl_2$.

100: 175060e **Substituted pyridinyl phenyl ethers and their pesticidal use.** Foerster, Heinz; Klauke, Erich; Priesnitz, Uwe; Riobel, Hans Jochem; Eue, Ludwig; Schmidt, Robert Rudolf (Bayer A.-G.) **Ger. Offen. DE 3,221,214** (Cl. C07D213/64), 08 Dec 1983, Appl. 04 Jun 1982; 64 pp. Title compds. (I) [R = CF_3,



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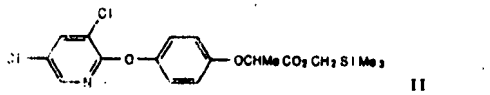


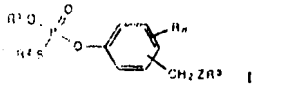
II

Cl; R^1 = H,Cl; R^2 = H,Me; R^3 = CHO, $CH(OR^4)_2$, $CO_2(CR^5R^6)_m(CR^7R^8)_nR^9$; R^4 = C_{1-4} alkyl or $(R^4R^4$ =) C_{2-3} alkylene; R^{5-8} = H, C_{1-4} alkyl; m = 1 or 2, n = 0 or 1; R^9 = Me_3Si, (un)substituted azolyl, phosphoryl, etc.] were prepd., as herbicides, by treatment of the appropriate acid, acid chloride, or phenol with the appropriate esterifying or etherifying agent. Thus, the corresponding acid and Me_3SiCH_2OH in the presence of 1,5-diazabicyclo[5.4.0]undecene gave (trimethylsilyl)methyl 2-[4-(3,5-dichloro-2-pyridyl)oxy]phenoxy]propionate (II) which was a more selective herbicide than 4-(2,4-$Cl_2C_6H_3O$)$C_6H_4OCHMeCO_2Me$.

100: 175061f **Organic silicates.** Matsumoto Seiyaku Kogyo Co., Ltd. **Jpn. Kokai Tokkyo Koho JP 58,216,194 [83,216,194]** (Cl. C07F7/08), 15 Dec 1983, Appl. 82/99,569, 10 Jun 1982; 5 pp. Hardening org. silicates were composed of ≥2 SiOR (R = satd. or unsatd. org. radical), ≥2 SiNCO, and optional metal chelates, alkoxides or acylates. Thus, a mixt. of 10 wt. parts $Si(OEt)_4$ and 2 wt. parts $Si(NCO)_4$ was stirred at room temp. under N to give a colorless liq., which showed no color charge in 3 mo and hardened to a colorless film in 15 min.

100: 175062g **Pharmacologically active bisphosphonates and pharmaceuticals containing these substances.** Instituto Gentili S.p.A. **Belg. BE 896,453** (Cl. C07F), 01 Aug 1983, IT Appl. 82/20,781, 15 Apr 1982; 24 pp. Phosphonylation of $H_2N(CH_2)_nCO_2H$ (n = 3, 4) gave $H_2N(CH_2)_nC[P(O)(OH)_2]_2OH$ (I). Thus, $H_2N(CH_2)_4CO_2H$ 9.4, $P(OH)_3$ 9.9, and $POCl_3$ 16.5 in PhCl gave I (n = 4) 12.4 kg. $H_2C[P(O)(OH_2]_2$ was also prepd. I inhibited urinary calculus. The LD_{50} for I (n = 3) was >2,000 mg/kg per os in mice.

100: 175063h **Thiophosphoric acid esters and their use.** Sehring Richard; Buck, Wolfgang; Prokic-Immel, Ricarda; Lust, Sigmund (Celamerck G.m.b.H. und Co. K.-G.) **Ger. Offen. DE 3,223,949** (Cl. C07F9/16), 29 Dec 1983, Appl. 26 Jun 1982; 16 pp. Thiophosphates



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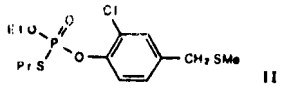
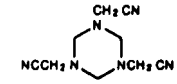


II

I (R = Cl, Br, F; R^1, R^2 = C_{1-3} alkyl; R^3 = C_{1-8} alkyl; Z = S, O; n = 1,2), useful as insecticides and acaricides, were prepd. Treating 2,4-$Cl(ClCH_2)C_6H_3OAc$ in MeOH with NaSMe at 5–10° and keeping 3 h at 50° gave 85% 2,4-$Cl(MeSCH_2)C_6H_3OH$ which was esterified with ClP(O)(OEt)SPr in PhMe previously treated with NEt_3 at 40°, then 4 h at 60° to give II. At 100 ppm, II killed 100% *Plutella maculipennis* and *Prodenia litura* and 99% *Tetranychus urticae*.

100: 175064j **N-Phosphonomethylglycine.** Felix, Raymond Anthony (Stauffer Chemical Co.) **Eur. Pat. Appl. EP 97,522** (Cl. C07F9/38), 04 Jan 1984, US Appl. 391,033, 22 Jun 1982; 14 pp.



II

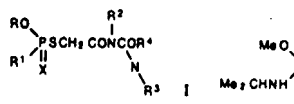
$HO_2CCH_2NHCH_2P(O)(OH)_2$ (I) was prepd. from triazine II. Thus II was treated with AcCl to give 79.85% $NCCH_2NAcCH_2Cl$ which was treated with $(MeO)_3P$ to give 79.32% $NCCH_2NAcCH_2P(O)(OMe)_2$ (III). Acid hydrolysis of III gave 35.49% I.

100: 175065k **Tertiary haloalkylphosphine oxides.** Lee, Fu Tseng Huang (FMC Corp.) **Eur. Pat. Appl. EP 98,251** (Cl. C07F9/53), 11 Jan 1984, US Appl. 392,901, 28 Jun 1982; 15 pp. Tertiary haloalkylphosphine oxides were prepd. by oxidative halogenation of tertiary hydroxyalkylphosphines. Thus $[HO(CH_2)_3]_3P$ was treated with HCl gas to give chlorinated $[HO(CH_2)_3]_3PO$ as the major product.

100: 175066m **N-phosphonomethylglycine derivatives and herbicidal compounds and compositions.** Bakel, Izhak (Geshuri Laboratories Ltd.) **Eur. Pat. Appl. EP 98,034** (Cl. C07F9/38), 11 Jan 1984, IL Appl. 66,137, 25 Jun 1982; 29 pp. $HO_2CCH_2NHC(=NH)NHR^1$.$H_2P(O)(OH)_2$ (I, R = Ph, alkylphenyl, halophenyl) were prepd. by oxidizing $(HO_2CCH_2)_2NCH_2P(O)(OH)_2$.$RNHC(NH)NHR^1$ (II). Thus II (R = R^1 = Ph) was oxidized with 30% H_2O_2 at 70% for 2 h to give I (R = R^1 = Ph) quant.

100: 175067n **2,2-Bis(haloalkenyl)-1-substituted-1-dialkoxyphosphoroethylene fungicides.** Ho, Andrew W. (Chevron Research Co.) **U.S. US 4,427,667** (Cl. 424-210; A01N57/18), 24 Jan 1984, Appl. 361,651, 25 Mar 1982; 9 pp. $RO(R^1O)P(X)CR^2$:C(SR^3)SR^4 (R, R^1 = alkyl; R^2 = cyano, alkoxycarbonyl; R^3, R^4 = alkenyl, haloalkenyl; X = O, S) were prepd. Thus $(EtO)_2P(O)CH_2CN$ was treated with CS_2 and *cis*-$ClCH_2CH$:CHCl to give $(EtO)_2P(O)C$:$(CN)C(SCH_2CH$:$CHCl)_2$ which at 250 ppm gave 63% inhibition of *Plasmopora viticola* on *Vitis vinifera*.

100: 175068p **(Di)thiophosphoric and phosphoric acid derivatives, compositions containing them and their plant protective use.** Staehler, Gerhard; Knauf, Werner; Sachse, Burkhard; Waltersdorfer, Anna (Hoechst A.-G.) **Ger. Offen. DE 3,228,631** (Cl. C07F9/24), 02 Feb 1984, Appl. 31 Jul 1982; 25 pp. (Amidocarbamoyl)methyl



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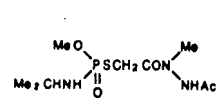
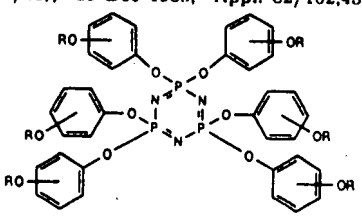


II

thiophosphonates and -phosphates I (R = C_{1-4} alkyl, R^1 = C_{1-4} alkyl, alkoxy, alkylthio, alkyl- or dialkylamino; R^2, R^3 = C_{1-4} alkyl, C_{5-6} cycloalkyl, benzyl, furylmethyl; R^1 = C_{1-4} alkyl, C_{1-3} alkoxymethyl or alkylthiomethyl; X = O or S) were prepd. (97 in all) and shown to have fungicidal and insecticidal activity. Thus, 18 g $Me_2CHNH(MeO)P(O)SK$ and 14 g $ClCH_2CONMeNHAc$ in 150 mL MeCN were refluxed 10 min to give the ester II.

100: 175069q **Bromine-substituted triaryl phosphates.** Bogach, E. V.; Zhuk, R. V.; Uskach, Ya. L.; Chekmareva, T. I.; Ermilina, N. I. **U.S.S.R. SU 1,065,419** (Cl. C07F9/12), 07 Jan 1984, Appl. 3,399,453, 19 Feb 1982. From *Otkrytiya, Izobret., Prom. Obraztsy, Tovarnye Znaki* 1984, (1), 106. The title compds. are prepd. by brominating triaryl phosphates with Br with heating. The uptake of Br is increased by brominating in a bromide–bromate medium at a (2.3–9.0):1 molar ratio of Br and Br salts in the presence of bipyridyl as a catalyst at 65–100°, and the spent bromide–bromate mixt. is returned to the process after reinforcing with bromates.

100: 175070h **Phosphonitrilic acid esters.** Otsuka Chemical Co., Ltd. **Jpn. Kokai Tokkyo Koho JP 58,219,190 [83,219,190]** (Cl. C07F9/22), 20 Dec 1983, Appl. 82/102,436, 15 Jun 1982; 11 pp.

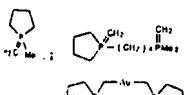


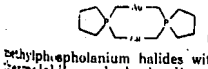
I

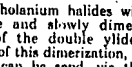
II

The title esters, e.g., I (R = H), were prepd. by esterification of halides II (X = halo) with $MeOC_6H_4OM$ (M = Na, K) followed by hydrolysis of the resultant I (R = Me) with pyridine HX salts. Thus, a soln. of 1 mol II (X = Cl) in THF was stirred with a soln. of 2.4 mol *p*-$MeOC_6H_4OH$ and 2.2 mol Na in THF at room temp. to 30° and THF distd. to give 98% I (R = Me at 4-position), which (0.3 mol) was heated with excess pyridine HCl at 205–210° to give 82% I (R = H at 4-position), which was treated with epichlorohydrin to give 91.5% hexaglycidyl ether (I; R = glycidyl at 4-position).

100: 175071j **Trialkylphosphines.** Nippon Chemical Industrial Co., Ltd. **Jpn. Kokai Tokkyo Koho JP 58,222,097 [83,222,097]**



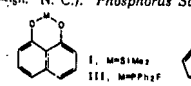




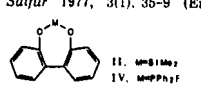
methylphospholanium halides with $NaNH_2$ in boiling THF, is thermolabile and slowly dimerizes with ring opening and formation of the double ylides II and III. Due to the reversibility of this dimerization, II and III are not obtained free from I but can be separ. via the corresponding phosphonium salts. The structure of I was supported by spectral data and an x-ray structure anal. of the gold complex IV.

88: 170243r **Compounds structurally related to complexones. Part V. Preparation and characterization of a new ligand phenylphosphinediacetic acid.** Podlahova, Jana (Dep. Inorg. Chem., Charles Univ., Prague, Czech.). *Collect. Czech. Chem. Commun.* 1978, 43(1), 57-63 (Eng). $PhP(CH_2CO_2H)_2$ was prepd. by reaction of the Reformatsky reagent with $PhPCl_2$, followed by hydrolysis of the ester formed. The reagent was prepd. in situ from Zn and $BrCH_2CO_2Et$. E. Erdos

88: 170244s **Some monocyclic and spirocyclic fluorophosphoranes containing 6- and 7-membered rings.** Littlefield, J. B.; Doak, G. O. (Dep. Chem., North Carolina State Univ., Raleigh, N. C.). *Phosphorus Sulfur* 1977, 3(1), 35-9 (Eng).



I, M=SiMe$_2$
III, M=PPh$_2$F



II, M=SiMe$_2$
IV, M=PPh$_2$F

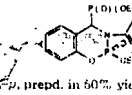
The reaction of dioxysilenin (I) and dioxysilepin (II) with PF_5 gave the expected monocyclic fluorophosphoranes (III) and (IV). Reaction of the same siloxy compds. with PF_5 gave products which could not be purified but whose ^{19}F NMR spectra suggested that the desired spirocyclic compds. were present in the impure products. Reaction of the siloxy compds. with $PhPF_4$ did not yield identifiable products.

88: 170245t **Reactions of bis(β-chloroethyl) vinylphosphonate with acids.** Nasakin, O. E.; Kormachev, V. V.; Stepanova, A. A.; Abramov, I. A.; Kukhtin, V. A. (Chuv. Gos. Univ. im. Yakovleva, Cheboksary, USSR). *Deposited Doc.* 1976, VINITI 1813-76, 5 pp. (Russ). Avail. VINITI. $(ClCH_2CH_2O=)_2P(O)CH_2CH_2O_2CR$ (R = o-, p-BrC_6H_4, 2-$C_{10}H_7CH_2$, o-$O_2N=C_6H_4CH_2$, $FSCH_2$, $ClCH_2$, $CH_2{:}CH$, $CH_2{:}CMe$, $PhCH{:}CH$, Me) were prepd. in 65-91% yields by heating $(ClCH_2CH_2O)_2P(O)CH{:}CH_2$ with RCO_2H at 60° 25 h.

88: 170246u **Synthesis and acid-base and complexing properties of amino-substituted α-hydroxyalkylidenediphosphonic acids.** Kabachnik, M. I.; Medved, T. Ya.; Dyatlova, N. M.; Tsvetkov, Yu. M.; Shcherbakov, B. K.; Bel'skii, F. I. (Inst. Elementoorg. Soedin., Moscow, USSR). *Izv. Akad. Nauk SSSR, Ser. Khim.* 1978, (2), 433-7 (Russ). $RR^1N(CH_2)_nC(O=P(O)(OH)_2)_2$ (I, R = R^1 = H, Me, Et, n = 2; R = R^1 = H, n = 3; R = H, R^1 = Et, n = 2) were prepd. in 46-57% yields. Thus, heating $H_2N(CH_2)_2CO_2H$ with H_3PO_3 and PCl_3 in PhCl at 100° 3 h gave 51% I (R = R^1 = H, n = 2). Their acid dissocn. consts. and the stability consts. of their complexes with Ca^{2+}, Co^{2+} and Cu^{2+} were detd.

88: 170247v **Synthesis and structure of O-alkyl hydrogen alkylselenophosphonates and their salts.** Nuretdinov, I. A.; Fedina, N. A.; Bayandina, E. V.; Loginova, E. I.; Giniyatullina, G. A. (Inst. Org. Khim. im. Arbuzova, Kazan, USSR). *Izv. Akad. Nauk SSSR, Ser. Khim.* 1978, (2), 437-40 (Russ). $R^1R^2P(Se)OH$ (R = Me, Et; R^1 = Et, Pr, Me_2CH, Bu, Me_2CHCH_2) (7 compds.) were prepd. in 51.7-65.7% yields by treating $R(R^1O)P(O)H$ with Se in presence of R^1ONa or hydroxylamine followed by acidification with HCl.

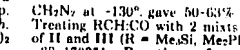
88: 170248w **Synthesis of 4-phospho-1,3,2-oxaaza-5,6-benzophosphorinanes.** Zeleneva, T. P.; Patlina, S. I.; Efanov, E. E. (USSR). *Zh. Obshch. Khim.* 1978, 48(2), 253-6 (Russ). Cyclocondensation of o-$C_6H_4CH[P(O)(OEt)_2]$=NC$_6$H$_4$NO$_2$-p, prepd. in 50% yield from o-$C_6H_4CH{:}NC_6H_4NO_2$-p



I

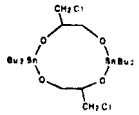
X = Cl, Br, I; Ar = 4-$MeOC_6H_4$, 4-ClC_6H_4, 4-$O_2NC_6H_4$, X = Cl were prepd. These adducts are thermally stable at ambient temp. but decomp. on exposure to atm. moisture at varying rate (Cl >> Br; AsX_3B >> $ArAsX_2$(mtn)). The adducts are noncondr dissocd. in soln. to varying degrees, the order of acceptor ability toward thioureas is $AsBr_3$ > $AsCl_3$, $ArAsI_2$, $ArAsBr_2$ > $ArAsCl_2$. Trigonal-bipyramidal structures are proposed for the adducts based on vibrational spectra. Secondary bonding leading to loosely joined dimeric mol. may occur as found by x-ray crystallog. for $AsCl_3$dmi.

88: 170250r **O- and C-organometallic isomers. XXI. Reaction of silyl and germyl ketenes with diazomethane.** Zaitseva, G. S.; Bogdanova, G. S.; Baukov, Yu. I.; Lutsenko, I. F. (Mosk. Gos. Univ., Moscow, USSR). *Zh. Obshch. Khim.* 1978, 48(1), 131-7 (Russ). Reaction of RCH:CO with 1 mol CH_2N_2 at -130° gave 50-63% I (R = Me_3Si, Me_3Ge, Et_3Ge). Treating RCH:CO with 2 mols CH_2N_2 gave 53-90% of a mixt. of II and III (R = Me_3Si, Me_2PhSi, Et_3Ge).



I II III

88: 170251s **Reactions of α-oxides with organotin acylates. II. Reaction of epichlorohydrin with dibutyltin disalicylate.** Klebanov, M. S.; Shoshina, L. V.; Shologon, I. M.; Nikonova, L. P. (Ukr. Nauchno-Issled. Inst. Plast., USSR). *Zh. Obshch. Khim.* 1978, 48(1), 138-41 (Russ). Treating epichlorohydrin

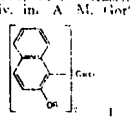


II

with dibutyltin disalicylate gave a mixt. of [o-$ClCH_2CH(OH)CH_2OC_6H_4CO_2]_2SnBu_2$ and [o-$ClCH_2CH(OH)CH_2O_2CC_6H_4O]_2SnBu_2$ (I). I reacts further with epichlorohydrin and H_2O to give II and o-$ClCH_2CH(OH)CH_2O_2CC_6H_4OH$.

88: 170252t **Reactions of $Ph_3SnCH_2CH_2NMe_2$ with electrophilic reagents.** Wardell, J. L. (Dep. Chem., Univ. Aberdeen, Aberdeen, Scot.). *Inorg. Chim. Acta* 1978, 26(1), L16 (Engl). Treating $Ph_3SnCH_2CH_2NMe_2$ with electrophiles either displaces Ph from the Sn, or causes decompn. of the mol. Thus $Ph_3SnCH_2CH_2NMe_2$ and EY (EY = $HgCl_2$, Br, Iodine) gave $Ph_2SnYCH_2CH_2NMe_2$ and PhE. Conversely, treating $Ph_3SnCH_2CH_2NMe_2$ with EY (EY = p-$MeC_6H_4SO_2Cl$, o-$O_2NC_6H_4SCl$, AcCl, MeI) gave Ph_2SnY_2, ethylene, and $ENMe_2$.

88: 170253u **Organogermanium compounds with α-alkoxy-α-naphthyl radicals.** Dumler, V. A.; Pomerantseva, L. V.; Gribanova, N. V.; Mazitkina, at. V. (Perm. Gos. Ped. Inst., USSR). *Org. Khim.* 1976, 56-60 (Russ). Edited by Yagupol'skii, I. Permsk. Gos. Univ. im. A. M. Gor'kogo: Perm', USSR.



I

Alkoxynaphthylgermane oxides I (R = Me, Et, Pr, Me_2CH, Bu, Me_2CHCH_2, $Me_2CHCH_2CH_2$, $Me(CH_2)_6$, n = 4-6) were prepd in 32-52% yields by treating β-alkoxy α-naphthylmagnesium bromides with $GeBr_4$ followed by hydrolysis with HBr.

88: 170254v **Reactivity of organotin compounds. XI. Alkyldemetalation of $RSn(CH_2)^-$ compounds.** Reutov, O. A.; Khutoryanskii, V. A.; Margorskova, O. I.; Beletskaya, I. P.; Reutov, O. A. (USSR). *Zh. Org. Khim.* 1978, 14(2), 265-6 (Russ). The ease of destannylation of $RSnMe_3$ by MeX^+ (X^+ = $O_3SC_6H_4Me$-4, OSO_2Me) in ($Me_2N)_3PO$ or 1:1 THF-Et_2O to give RMe and Me_3SnX decreased in the order R = 9-cyano-9-fluorenyl >> 1-indenyl > 3-methyl-1-indenyl >> 9-fluorenyl. In the presence of Me_3SnR (R = 1-indenyl) in $(Me_2N)_3PO$, 1-methylindene isomerized to 3-methylindene. The reactions took place via formation of an $[R]^+$ $[SnMe_3]^-$ ion pair.

88: 170255w **New heterocyclic ring system containing arsenic: synthesis of 1-chloro-3,4-dihydro-1H-[1,3,2]oxarsenino[2,3-b]benzoxarsenin.** Bhatia, M. S.; Gill, U. S. (Punjab Agric. Univ., Ludhiana, India). *Indian J. Chem., Sect. B* 1977, 15B(12), 1145-1146 (Eng). The title compd. (I) was prepd.

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